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(54) Title: MULTIPLE-TUMOR ABERRANT GROWTH GENES

(57) Abstract

The present invention relates to the multi-tumor Aberrant Growth (MAG) gene having the nucleotide sequence of any one of the strands of any one of the members of the High Mobility Group protein genes or LIM protein genes, including modified versions thereof. The gene and its derivatives may be used in various diagnostic and therapeutic applications.

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MULTIPLE-TUMOR ABERRANT GROWTH GENES

The present invention relates to the
identification of the High Mobility Group (HMG) protein gene
5 family as a family of genes frequently associated with
aberrant cell growth as found in a variety of both benign
and malignant tumors. The invention in particular relates to
the identification of a member of the HMG gene family as the
broadly acting chromosome 12 breakpoint region gene involved
10 in a number of tumors, including but not limited to the
mesenchymal tumors hamartomas (e.g. breast and lung),
lipomas, pleomorphic salivary gland adenomas, uterine
leiomyomas, angiomyxomas, fibroadenomas of the breast,
polyps of the endometrium, atherosclerotic plaques, and
15 other benign tumors as well as various malignant tumors,
including but not limited to sarcomas (e.g.
rhabdomyosarcoma, osteosarcoma) and carcinomas (e.g. of
breast, lung, skin, thyroid), as well as leukemias and
lymphomas. The invention also relates to another member of
20 the HMG gene family that was found to be implicated in
breaks in chromosome 6.
Furthermore, the invention concerns the
identification of members of the LIM protein family as
another type of tumor-type specific breakpoint region genes
25 and frequent fusion partners of the HMG genes in these
tumors. The LPP (Lipoma-Preferred Partner) gene of this
family is found to be specific for lipomas. The invention
relates in particular to the use of the members of the HMG
and LIM gene family and their derivatives in diagnosis and
30 therapy.
Multiple independent cytogenetic studies have
firmly implicated region q13-q15 of chromosome 12 in a
variety of benign and malignant solid tumor types. Among
benign solid tumors, involvement of 12q13-q15 is frequently
35 observed in benign adipose tissue tumors [1], uterine
leiomyomas [2, 3], and pleomorphic adenomas of the salivary
glands [4, 5]. Involvement of the same region has also been
reported for endometrial polyps [6, 7] for hemangio-

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pericytoma [8], and for chondromatous tumors [9, 10, 11, 12]. Recently, the involvement of chromosome 12q13-q15 was reported in pulmonary chondroid hamartoma [13, 14]. Finally, several case reports of solid tumors with involvement of

5 chromosome region 12q13-q15 have been published; e.g. tumors of the breast [15, 16], diffuse astrocytomas [17], and a giant-cell tumor of the bone [18]. Malignant tumor types with recurrent aberrations in 12q13-q15 include myxoid liposarcoma [19], soft tissue clear-cell sarcoma [20, 21,

10 22], and a subgroup of rhabdomyosarcoma [23].

Although these studies indicated that the same cytogenetic region of chromosome 12 is often involved in chromosome aberrations, like translocations, in these solid tumors, the precise nature of the chromosome 12 breakpoints

15 in the various tumors is still not known. Neither was it established which genes are affected directly by the translocations.

In previous physical mapping studies [39], the chromosome 12q breakpoints in lipoma, pleomorphic salivary

20 gland adenoma, and uterine leiomyoma were mapped between locus D12S8 and the CHOP gene and it was shown that D12S8 is located distal to CHOP. Recently, it was also found by FISH analysis that the chromosome 12q breakpoints in a hamartoma of the breast, an angiomyxoma and multiple pulmonary

25 chondroid hamartomas are mapping within this DNA interval. In an effort to molecularly clone the genes affected by the chromosome 12q13-q15 aberrations in the various tumors, the present inventors chose directional chromosome walking as a structural approach to define the DNA region encompassing

30 these breakpoints.

As a starting point for chromosome walking, locus D12S8 was used. During these walking studies, it was shown that the chromosomal breakpoints as present in a number of uterine leiomyoma-derived cell lines are clustered within a

35 445 kb chromosomal segment which has been designated Uterine Leiomyoma Cluster Region on chromosome 12 (ULCR12) [24]. Subsequently, it was found that a 1.7 Mb region on chromosome 12 encompasses the chromosome 12 breakpoints of

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uterine leiomyoma-, lipoma-, and salivary gland adenoma-
cells, with the breakpoint cluster regions of the various
tumor types overlapping [25, "ANNEX 1"]. This 1.7 Mb region
on the long arm of chromosome 12, which contains ULCR12
5 obviously, was designated Multiple Aberration Region (MAR)
to reflect this feature. In a regional fine mapping study,
MAR was recently assigned to 12q15.
        It has thus been found that essentially all
breakpoints of chromosome 12 map in a 1.7 Mb region
10 indicated herein as the "Multiple Aberration Region" or MAR.
Further research revealed that in this region a member of
the High Mobility Group gene family, the HMGI-C gene, can be
identified as a postulated multi-tumor aberrant growth gene
(MAG). The same applies to members of the LIM family which
15 are also found to be involved in chromosome aberrations. Of
these the chromosome 3-derived Lipoma-Preferred Partner
(LPP) gene is particularly implicated in lipomas.
        LIM proteins are proteins carrying cystein-rich
zinc-binding domains, so-called LIM domains. They are
20 involved in protein-protein interactions [for a review see
ref. 80]. One of the members of the protein family is the
now disclosed LPP protein mapping at chromosome 3.
        According to the present invention the aberrations
in the HMGI-C gene on chromosome 12 and the LPP gene on
25 chromosome 3 have been used as a model to reveal the more
general concept of the involvement of members of the HMG and
LIM gene families in both benign and malignant tumors. To
demonstrate that there exists a more general concept of gene
families, which, when affected by chromosome rearrangements,
30 lead to a particular group of tumor growth, the present
inventors demonstrated that the HMGI(Y) gene, which is a
member of the HMG family, is involved in breaks in
chromosome 6.
        Although both the HMG and LIM gene families are
35 known per se, up till the present invention the correlation
between these families and tumor inducing chromosome
aberrations, like translocations, deletions, insertions and
inversions, has not been anticipated. Furthermore, until now

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it was not previously demonstrated that alterations in the
physiological expression level of the members of the gene
family are probably also implicated in tumor development.
According to the invention it was demonstrated that in
5 normal adult cells the expression level of the HMGI-C gene
is practically undetectable, whereas in aberrantly growing
cells the expression level is significantly increased.

Based on these insights the present invention now
provides for the members of the gene families or derivatives
10 thereof in isolated form and their use in diagnostic and
therapeutic applications. Furthermore the knowledge on the
location and nucleotide sequence of the genes may be used to
study their rearrangements or expression and to identify a
possible increase or decrease in their expression level and
15 the effects thereof on cell growth. Based on this
information diagnostic tests or therapeutic treatments may
be designed.

In this application the term "Multi-tumor Aberrant
Growth (or MAG) gene" will be used to indicate the
20 involvement of these types of genes in various types of
cancer. The term refers to all members of the HMG and LIM
gene families involved in non-physiological proliferative
growth, and in particular involved in malignant or benign
tumors, including atherosclerotic plaques. However,
25 according to the invention it was furthermore found that
even breaks outside the actual gene but in the vicinity
thereof might result in aberrant growth. The term MAG gene
is therefore also intended to include the immediate vicinity
of the gene. The skilled person will understand that the
30 "immediate vicinity" should be understood to include the
surroundings of the gene in which breaks or rearrangements
will result in the above defined non-physiological
proliferative growth.

The term "wildtype cell" is used to indicate the
35 cell not harbouring an aberrant chromosome or to a cell
having a physiological expression level of the relevant
gene. "Wildtype" or "normal" chromosome refers to a non-
aberrant chromosome.

The present invention provides for various
diagnostic and therapeutic applications that are based on
the information that may be derived from the genes. This
information not only encompasses its nucleotide sequence or
5 the amino acid sequence of the gene product derived from the
gene, but also involves the levels of transcription or
translation of the gene.

The invention is thus two-fold. On the one hand
the aberration in cell growth may be directly or indirectly
10 caused by the physical breaks that occur in the gene or its
vicinity. On the other hand the aberration in cell growth
may be caused by a non-physiological expression level of the
gene. This non-physiological expression level may be caused
by the break, or may be due to another stimulus that
15 activates or deactivates the gene. At present the exact
mechanism or origin of the aberrant cell growth is not yet
unraveled. However, exact knowledge on this mechanism is not
necessary to define methods of diagnosis or treatment.

Diagnostic methods according to the invention are
20 thus based on the fact that an aberration in a chromosome
results in a detectable alteration in the chromosomes'
appearance or biochemical behaviour. A translocation, for
example will result in a first part of the chromosome (and
consequently of the MAG gene) having been substituted for
25 another (second) part (further referred to as "first and
second substitution parts"). The first part will often
appear someplace else on another chromosome from which the
second part originates. As a consequence hybrids will be
formed between the remaining parts of both (or in cases of
30 triple translocations, even more) chromosomes and the
substitution parts provided by their translocation partners.
Since it has now been found that the breaks occur in a MAG
gene this will result in hybrid gene products of that MAG
gene. Markers, such as hybridising molecules like RNA, DNA
35 or DNA/RNA hybrids, or antibodies will be able to detect
such hybrids, both on the DNA level, and on the RNA or
protein level.

For example, the transcript of a hybrid will still
comprise the region provided by the remaining part of the
gene/chromosome but will miss the region provided by the
substitution part that has been translocated. In the case of
5 inversions, deletions and insertions the gene may be equally
afflicted.

Translocations are usually also cytogenetically
detectable. The other aberrations are more difficult to find
because they are often not visible on a cytogenetical level.
10 The invention now provides possibilities for diagnosing all
these types of chromosomal aberrations.

In translocations markers or probes based on the
MAG gene for the remaining and substitution parts of a
chromosome _in situ_ detect the remaining part on the original
15 chromosome but the substitution part on another, the
translocation partner.

In the case of inversions for example, two probes
will hybridise at a specific distance in the wildtype gene.
This distance might however change due to an inversion. _In_
20 _situ_ such inversion may thus be visualized by labeling a set
of suitable probes with the same or different detectable
markers, such as fluorescent labels. Deletions and
insertions may be detected in a similar manner.

According to the invention the above _in situ_
25 applications can very advantageously be performed by using
FISH techniques. The markers are e.g. two cosmids one of
which comprises exons 1 to 3 of the MAG gene, while the
other comprises exons 4 and 5. Both cosmids are labeled with
different fluorescent markers, e.g. blue and yellow. The
30 normal chromosome will show a combination of both labels,
thus giving a green signal, while the translocation is
visible as a blue signal on the remaining part of one
chromosome (e.g. 12) while the yellow signal is found on
another chromosome comprising the substitution part. In case
35 the same labels are used for both probes, the intensity of
the signal on the normal chromosome will be 100%, while the
signal on the aberrant chromosomes is 50%. In the case of

inversions one of the signals shifts from one place on the
normal chromosome to another on the aberrant one.

In the above applications a reference must be
included for comparison. Usually only one of the two
5 chromosomes is afflicted. It will thus be very convenient to
use the normal chromosome as an internal reference.
Furthermore it is important to select one of the markers on
the remaining or unchanging part of the chromosome and the
other on the substitution or inverted part. In the case of
10 the MAG gene of chromosome 12, breaks are usually found in
the big intron between exons 3 and 4 as is shown by the
present invention. Furthermore breaks were detected between
exons 4 and 5. Probes based on exons 1 to 3 and 4 and 5, or
probes based on either exon 4 or on exon 5 are thus very
15 useful. As an alternative a combination of probes based on
both translocation or fusion partners may be used. For
example, for the identification of lipomas one may use
probes based on exons 1 to 3 of the HMGI-C gene on the one
hand and based on the LIM domains of the LPP gene on the
20 other hand.

Furthermore it was found that breaks might also
occurr outside the gene, i.e. 5' or 3' thereof. The choice
of the probes will then of course include at least one probe
hybrising to a DNA sequence located 5' or 3' of the gene.
25   "Probes" as used herein should be widely
interpreted and include but are not limited to linear DNA or
RNA strands, Yeast Artificial Chromosomes (YACs), or
circular DNA forms, such as plasmids, phages, cosmids etc..

These in situ methods may be used on metaphase and
30 interphase chromosomes.

Besides the above described in situ methods
various diagnostic techniques may be performed on a more
biochemical level, for example based on alterations in the
DNA, RNA or protein, or on changes in the physiological
35 expression level of the gene.

Basis for the methods that are based on
alterations in the chromosome's biochemical behaviour is the
fact that by choosing suitable probes, variations in the

length or composition in the gene, transcript or protein may
be detected on a gel or blot. Variations in length are
visible because the normal gene, transcript(s) or protein(s)
will appear in another place on the gel or blot then the

5 aberrant one(s). In case of a translocation more than the
normal number of spots will appear.

Based on the above principle the invention may
thus for example relate to a method of diagnosing cells
having a non-physiological proliferative capacity,

10 comprising the steps of taking a biopsy of the cells to be
diagnosed, isolating a suitable MAG gene-related
macromolecule therefrom, and analysing the macromolecule
thus obtained by comparison with a reference molecule
originating from cells not showing a non-physiological

15 proliferative capacity, preferably from the same individual.
The MAG gene-related macromolecule may thus be a DNA, an RNA
or a protein. The MAG gene may be either a member of the HMG
family or of the LIM family.

In a specific embodiment of this type of

20 diagnostic method the invention comprises the steps of
taking a biopsy of the cells to be diagnosed, extracting
total RNA thereof, preparing a first strand cDNA of the mRNA
species in the total RNA extract or poly-A-selected
fraction(s) thereof, which cDNA comprises a suitable tail;

25 performing a PCR using a MAG gene specific primer and a
tail-specific primer in order to amplify MAG gene specific
cDNA's; separating the PCR products on a gel to obtain a
pattern of bands; evaluating the presence of aberrant bands
by comparison to wildtype bands, preferably originating from

30 the same individual.

As an alternative amplification may be performed
by means of the Nucleic Acid Sequence-Based Amplification
(NASBA) technique [81] or variations thereof.

In another embodiment the method comprises the

35 steps of taking a biopsy of the cells to be diagnosed,
isolating total protein therefrom, separating the total
protein on a gel to obtain essentially individual bands,
optionally transfering the bands to a Western blot,

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hybridising the bands thus obtained with antibodies directed
against a part of the protein encoded by the remaining part
of the MAG gene and against a part of the protein encoded by
the substitution part of the MAG gene; visualising the
5 antigen-antibody reactions and establishing the presence of
aberrant bands by comparison with bands from wildtype
proteins, preferably originating from the same individual.

In a further embodiment the method comprises
taking a biopsy of the cells to be diagnosed; isolating
10 total DNA therefrom; digesting the DNA with one or more so-
called "rare cutter" restriction enzymes (typically "6- or
more cutters"); separating the digest thus prepared on a gel
to obtain a separation pattern; optionally transfering the
separation pattern to a Southern blot; hybridising the
15 separation pattern in the gel or on the blot with a set of
probes under hybridising conditions; visualising the
hybridisations and establishing the presence of aberrant
bands by comparison to wildtype bands, preferably
originating from the same individual.

20   Changes in the expression level of the gene may be
detected by measuring mRNA levels or protein levels by means
of a suitable probe.

Diagnostic methods based on abnormal expression
levels of the gene may comprise the steps of taking a sample
25 of the cells to be diagnosed; isolating mRNA therefrom; and
establishing the presence and/or the (relative) quantity of
mRNA transcribed from the MAG gene of interest in comparison
.to a control. Establishing the presence or (relative)
quantity of the mRNA may be achieved by amplifying at least
30 part of the mRNA of the MAG gene by means of RT-PCR or
similar amplification techniques. In an alternative
embodiment the expression level may be established by
determination of the presence or the amount of the gene
product (e.g. protein) by means of for example monoclonal
35 antibodies.

The diagnostic methods of the invention may be
used for diseases wherein cells having a non-physiological
proliferative capacity are selected from the group

consisting of benign tumors, such as the mesenchymal tumors
hamartomas (e.g. breast and lung), adipose tissue tumors
(e.g. lipomas), pleomorphic salivary gland adenomas, uterine
leiomyomas, angiomyxomas, fibroadenomas of the breast,
5   polyps of the endometrium, atherosclerotic plaques, and
other benign tumors as well as various malignant tumors,
including but not limited to sarcomas (e.g.
rhabdomyosarcoma, osteosarcoma) and carcinomas (e.g. of
breast, lung, skin, thyroid). The invention is not limited
10 to the diagnosis and treatment of so-called benign and
malignant solid tumors, but the principles thereof have been
found to also apply to haematological malignancies like
leukemias and lymphomas.

        Recent publications indicate that atherosclerotic
15 plaques also involve abnormal proliferation [26] of mainly
smooth muscle cells and it was postulated that
atherosclerotic plaques constitute benign tumors [27].
Therefore, this type of disorder is also to be understood as
a possible indication for the use of the MAG gene family, in
20 particular in diagnostic and therapeutic applications.

        As already indicated above it has been found that
in certain malignant tumors the expression level of the HMG
genes is increased [28]. Until now the relevance of this
observation was not understood. Another aspect of the
25 invention thus relates to the implementation of the
identification of the MAG genes in therapy. The invention
for example provides anti-sense molecules or expression
inhibitors of the MAG gene for use in the treatment of
diseases involving cells having a non-physiological
30 proliferative capacity by modulating the expression of the
gene. A non-physiological high expression may thus be
normalised by means of antisense RNA that is either
administered to the cell or expressed thereby and binds to
the mRNA, or antibodies directed to the gene product, which
35 in turn may result in a normalisation of the cell growth.
The examples will demonstrate that expression of antisense
RNA in leukemic cells results in a re-differentiation of the
cells back to normal.

The invention thus provides derivatives of the MAG gene and/or its immediate environment for use in diagnosis and the preparation of therapeutical compositions, wherein the derivatives are selected from the group consisting of
5 sense and anti-sense cDNA or fragments thereof, transcripts of the gene or fragments thereof, antisense RNA, triple helix inducing molecule or other types of "transcription clamps", fragments of the gene or its complementary strand, proteins encoded by the gene or fragments thereof, protein
10 nucleic acids (PNA), antibodies directed to the gene, the cDNA, the transcript, the protein or the fragments thereof, as well as antibody fragments. Besides the use of direct derivatives of the genes and their surroundings (flanking sequences) in diagnosis and therapy, other molecules, like
15 expression inhibitors or expression enhancers, may be used for therapeutic treatment according to the invention. An example of this type of molecule are ribozymes that destroy RNA molecules.

Besides the above described therapeutic and
20 diagnostic methods the principles of the invention may also be used for producing a transgenic animal model for testing pharmaceuticals for treatment of MAG gene related malignant or benign tumors and atherosclerotic plaques. One of the examples describes the production of such an animal model.

25   It is to be understood that the principles of the present invention are described herein for illustration purposes only with reference to the HMGI-C gene mapping at chromosome 12 and the HMGI(Y) gene mapping at chromosome 6 and the LPP gene on chromosome 3. Based on the information
30 provided in this application the skilled person will be able to isolate and sequence corresponding genes of the gene family and apply the principles of this invention by using the gene and its sequence without departing from the scope of the general concept of this invention.

35   The present invention will thus be further elucidated by the following examples which are in no way intended to limit the scope thereof.

EXAMPLES

EXAMPLE 1

1. Introduction

       This example describes the isolation and analysis

5 of 75 overlapping YAC clones and the establishment of a YAC
contig (set of overlapping clones), which spans about 6 Mb
of genomic DNA around locus D12S8 and includes MAR. The
orientation of the YAC contig on the long arm of chromosome
12 was determined by double-color FISH analysis. On the

10 basis of STS-content mapping and restriction enzyme
analysis, a long range physical map of this 6 Mb DNA region
was established. The contig represents a useful resource for
cDNA capture aimed at identifying genes located in 12q15,
including the one directly affected by the various

15 chromosome 12 aberrations.

2. Materials and methods

2.1. Cell lines

       Cell lines PK89-12 and LIS-3/SV40/A9-B4 were used

20 for Chromosome Assignment using Somatic cell Hybrids (CASH)
experiments. PK89-12, which contains chromosome 12 as the
sole human chromosome in a hamster genetic background, has
been described before [29]. PK89-12 cells were grown in DME-
F12 medium supplemented with 10% fetal bovine serum, 200

25 IU/ml penicillin, and 200 μg/ml streptomycin. Somatic cell
hybrid LIS-3/SV40/A9-B4 was obtained upon fusion of myxoid
liposarcoma cell line LIS-3/SV40, which carries a
t(12;16)(q13;p11.2), and mouse A9 cells and was previously
shown to contain der(16), but neither der(12) nor the normal

30 chromosome 12 [30]. LIS-3/SV40/A9-B4 cells were grown in
selective AOA-medium (AOA-medium which consisted of DME-F12
medium supplemented with 10% fetal bovine serum, 0.05 mM
adenine, 0.05 mM ouabain, and 0.01 mM azaserine). Both cell
lines were frequently assayed by standard cytogenetic

35 techniques.

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2.2. Nucleotide sequence analysis and oligonucleotides.

Nucleotide sequences were determined according to
the dideoxy chain termination method using a T7 polymerase
sequencing kit (Pharmacia/LKB) or a dsDNA Cycle Sequencing
5 System (GIBCO/BRL). DNA fragments were subcloned in pGEM-
3Zf(+) and sequenced using FITC-labelled standard SP6 or T7
primers, or specific primers synthesized based upon newly
obtained sequences. Sequencing results were obtained using
an Automated Laser Fluorescent (A.L.F.) DNA sequencer
10 (Pharmacia Biotech) and standard 30 cm, 6% Hydrolink[R], Long
Range[TM] gels (AT Biochem). The nucleotide sequences were
analyzed using the sequence analysis software Genepro
(Riverside Scientific), PC/Gene (IntelliGenetics), the
IntelliGenetics Suite software package (IntelliGenetics,
15 Inc.), and Oligo [31]. All oligonucleotides were purchased
from Pharmacia Biotech.

2.3. Chromosome preparations and fluorescence in situ
     hybridization (FISH)
20   FISH analysis of YAC clones was performed to
establish their chromosomal positions and to identify
chimeric clones. FISH analysis of cosmid clones
corresponding to STSs of YAC insert ends were performed to
establish their chromosomal positions. Cosmids were isolated
25 from human genomic library CMLW-25383 [32] or the arrayed
chromosome 12-specific library constructed at Lawrence
Livermore National Laboratory (LL12NC01, ref. 33) according
to standard procedures [34]. Routine FISH analysis was
performed essentially as described before [30, 35]. DNA was
30 labelled with biotin-11-dUTP (Boehringer) using the protocol
of Kievits et al. [36]. Antifade medium, consisting of DABCO
(2 g/100 ml, Sigma), 0.1 M Tris-HCL pH 8, 0.02% Thimerosal,
and glycerol (90%), and containing propidium iodide (0.5
μg/ml, Sigma) as a counterstain, was added 15 min before
35 specimens were analyzed on a Zeiss Axiophot fluorescence
microscope using a double band-pass filter for FITC/Texas
red (Omega Optical, Inc.). Results were recorded on Scotch
(3M) 640 ASA film.

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For the double colour FISH experiments,
LLNL12NCO1-96C11 was labelled with digoxygenin-11-dUTP
(Boehringer) and cosmids LLNL12NCO1-1F6 and -193F10, with
biotin-11-dUTP. Equal amounts of each probe were combined
5 and this mixture was used for hybridization. After
hybridization, slides were incubated for 20 min with Avidin-
FITC and then washed as described by Kievits et al. [36].
Subsequent series of incubations in TNB buffer (0.1 M Tris-
HCl pH 7.5, 0.15 M NaCl, 0.5% Boehringer blocking agent
10 (Boehringer)) and washing steps were performed in TNT buffer
(0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween-20); all
incubations were performed at 37 °C for 30 min. During the
second incubation, Goat-α-Avidin-biotin (Vector) and Mouse-
α-digoxygenin (Sigma) were applied simultaneously. During
15 the third incubation, Avidin-FITC and Rabbit-α-Mouse-TRITC
(Sigma) were applied. During the last incubation, Goat-α-
Rabbit-TRITC (Sigma) was applied. After a last wash in TNT
buffer, samples were washed twice in 1 x PBS and then
dehydrated through an ethanol series (70%, 90%, 100%).
20 Antifade medium containing 75 ng/μl DAPI (Serva) as
counterstain was used. Specimens were analyzed on a Zeiss
Axiophot fluorescence microscope as described above.

2.4. Screening of YAC libraries.
25   YAC clones were isolated from CEPH human genomic
YAC libraries mark 1 and 3 [37, 38] made available to us by
the Centre d'Étude du Polyphormisme Humain (CEPH). Screening
was carried out as previously described [39]. Contaminating
Candida parapsylosis, which was sometimes encountered, was
30 eradicated by adding terbinafin (kindly supplied by Dr.
Dieter Römer, Sandoz Pharma LTD, Basle, Switzerland) to the
growth medium (final concentration: 25 μg/ml). The isolated
YAC clones were characterized by STS-content mapping,
contour-clamped homogeneous electric field (CHEF) gel
35 electrophoresis [40], restriction mapping, and
hybridization- and FISH analysis.

2.5. PCR reactions

PCR amplification was carried out using a
Pharmacia/LKB Gene ATAQ Controller (Pharmacia/LKB) in final
volumes of 100 μl containing 10 mM Tris-HCl pH 8.3, 50 mM
5 KCl, 1.5 mM $MgCl_2$, 0.01% gelatine, 2 mM dNTP's, 20 pmole of
each amplimer, 2.5 units of Amplitaq (Perkin-Elmer Cetus),
and 100 ng (for superpools) or 20 ng (for pools) of DNA.
After initial denaturation for 5 min at 94 °C, 35
amplification cycles were performed each consisting of
10 denaturation for 1 min at 94 °C, annealing for 1 min at the
appropriate temperature (see Table I) and extension for 1
min at 72 °C. The PCR reaction was completed by a final
extension at 72 °C for 5 min. Results were evaluated by
analysis of 10 μl of the reaction product on polyacrylamide
15 minigels.

2.6. Pulsed-field gel electrophoresis and Southern blot analysis

Pulsed-field gel electrophoresis and Southern blot
20 analysis were performed exactly as described by Schoenmakers
et al. [39]. Agarose plugs containing high-molecular weight
YAC DNA (equivalent to about 1 x 10^8 yeast cells) were twice
equilibrated in approximately 25 ml TE buffer (pH 8.0) for
30 min at 50 °C followed by two similar rounds of
25 equilibration at room temperature. Plugs were subsequently
transferred to round-bottom 2 ml eppendorf tubes and
equilibrated two times for 30 min in 500 μl of the
appropriate 1 x restriction-buffer at the appropriate
restriction temperature. Thereafter, DNA was digested in the
30 plugs according to the suppliers (Boehringer) instructions
for 4 h using 30 units of restriction endonuclease per
digestion reaction. After digestion, plugs along with
appropriate molecular weight markers were loaded onto a 1%
agarose / 0.25 x TBE gel, sealed with LMP-agarose and size
35 fractionated on a CHEF apparatus (Biorad) for 18 h at 6.0
V/cm using a pulse angle of 120 degrees and constant pulse
times varying from 10 sec (separation up to 300 kbp) to 20
sec (separation up to 500 kbp). In the case of large

16

restriction fragments, additional runs were performed,
aiming at the separation of fragments with sizes above 500
kbp. Electrophoresis was performed at 14 °C in 0.25 x TBE.
As molecular weight markers, lambda ladders (Promega) and
5 home-made plugs containing lambda DNA cut with restriction
endonuclease HindIII were used. After electrophoresis, gels
were stained with ethidium bromide, photographed, and UV
irradiated using a stratalinker (Stratagene) set at 120 mJ.
DNA was subsequently blotted onto Hybond N$^+$ membranes
10 (Amersham) for 4-16 h using 0.4 N NaOH as transfer buffer.
After blotting, the membranes were dried for 15 min at 80
°C, briefly neutralised in 2 x SSPE, and prehybridised for
at least 3 h at 42 °C in 50 ml of a solution consisting of
50% formamide, 5 x SSPE, 5 x Denhardts, 0.1% SDS and 200
15 µg/ml heparin. Filters were subsequently hybridised for 16 h
at 42 °C in 10 ml of a solution consisting of 50% formamide,
5 x SSPE, 1 x Denhardts, 0.1% SDS, 100 µg/ml heparin, 0.5%
dextran sulphate and 2-3 x 10^6 cpm/ml of labelled probe.
Thereafter, membranes were first washed two times for 5 min
20 in 2 x SSPE/0.1% SDS at room temperature, then for 30 min in
2 x SSPE/0.1% SDS at 42 °C and, finally, in 0.1 x SSPE/0.1%
SDS for 20 min at 65 °C. Kodak XAR-5 films were exposed at -
80 °C for 3-16 h, depending on probe performance.
Intensifying screens (Kyokko special 500) were used.
25

2.7. Generation of STSs from YAC insert ends
        STSs from YAC insert ends were obtained using a
vectorette-PCR procedure in combination with direct DNA
sequencing analysis, essentially as described by Geurts et
30 al. [41]. Primer sets were developed and tested on human
genomic DNA, basically according to procedures described
above. STSs will be referred to throughout this application
by their abbreviated names (for instance: RM1 instead of STS
12-RM1) for reasons of convenience.
35

3. Results

3.1. Assembly of a YAC Contig around locus D12S8

In previous studies [39], a 800 kb YAC contig around D12S8 was described. This contig consisted of the
5 following three partially overlapping, non-chimeric CEPH YAC clones: 258F11, 320F6, and 234G11. This contig was used as starting point for a chromosome walking project to define the DNA region on the long arm of chromosome 12 that encompasses the breakpoints of a variety of benign solid
10 tumors, which are all located proximal to D12S8 and distal to CHOP. Initially, chromosome walking was performed bidirectionally until the size of the contig allowed reliable determination of the orientation of it. In the bidirectional and subsequent unidirectional chromosome
15 walking steps, the following general procedure was used. First, rescuing and sequencing the ends of YAC clones resulted in DNA markers characterizing the left and right sides of these (Table I). Based on sequence data of the ends of forty YAC inserts, primer sets were developed for
20 specific amplification of DNA; establishing STSs (Table II). Their localization to 12q13-qter was determined by CASH as well as FISH after corresponding cosmid clones were isolated. It should be noted that isolated YAC clones were often evaluated by FISH analysis too, thus not only
25 revealing the chromosomal origin of their inserts but also, for a number of cases, establishing and defining their chimeric nature. Moreover, it should be emphasized that data obtained by restriction endonuclease analysis of overlapping YAC clones were also taken into account in the YAC clone
30 evaluation and subsequent alignment. With the sequentially selected and evaluated primer sets, screening of the YAC and cosmid libraries was performed to isolate the building blocks for contig-assembly. Therefore, contig-assembly was performed using data derived from FISH- and STS-content
35 mapping as well as restriction endonuclease analysis. Using this approach, we established a YAC contig consisting of 75 overlapping YAC clones, covering approximately 6 Mb of DNA (Fig. 1). This contig appeared to encompass the chromosome

12 breakpoints of all tumor-derived cell lines studied [39].
Characteristics of the YACs that were used to build this
contig are given in Table I.

5 3.2. Establishment of the Chromosomal Orientation of the YAC
        Contig
            To allow unidirectional chromosome walking towards
    the centromere of chromosome 12, the orientation of the DNA
    region flanked by STSs RM14 and RM26 (approximate size: 1450
10 kb) was determined by double-color interphase FISH analysis.
    Cosmid clones corresponding to these STSs (i.e. LL12NC01-1F6
    (RM14) and LL12NC01-96C11 (RM26)) were differentially
    labelled to show green or red signals, respectively. As a
    reference locus, cosmid LL12NC01-193F10 was labelled to show
15 green signals upon detection. LL12NC01-193F10 had previously
    been mapped distal to the breakpoint of LIS-3/SV40 (i.e.
    CHOP) and proximal to the chromosome 12q breakpoints in
    lipoma cell line Li-14/SV40 and uterine leiomyoma cell line
    LM-30.1/SV40. LL12NC01-1F6 and LL12NC01-96C11 were found to
20 be mapping distal to the 12q breakpoints in lipoma cell line
    Li-14/SV40 and uterine leiomyoma cell line LM-30.1/SV40.
    Therefore, LL12NC01-193F10 was concluded to be mapping
    proximal to both RM14 and RM26 (unpublished results). Of 150
    informative interphases scored, 18% showed a signal-order of
25 red-green-green whereas 72% showed a signal order of green-
    red-green. Based upon these observations, we concluded that
    RM26 mapped proximal to RM14, and therefore we continued to
    extend the YAC contig from the RM26 (i.e. proximal) side of
    our contig only. Only the cosmids containing RM14 and RM26
30 were ordered by double-color interphase mapping; the order
    of all others was deduced from data of the YAC contig.
    Finally, it should be noted that the chromosomal orientation
    of the contig as proposed on the basis of results of the
    double-color interphase FISH studies was independently
35 confirmed after the YAC contig had been extended across the
    chromosome 12 breakpoints as present in a variety of tumor
    cell lines. This confirmatory information was obtained in
    extensive FISH studies in which the positions of YAC and

cosmid clones were determined relative to the chromosome
12q13-q15 breakpoints of primary lipomas, uterine
leiomyomas, pleomorphic salivary gland adenomas, and
pulmonary chondroid hamartomas or derivative cell lines [24,
5 42, 25, 43].

    3.3. Construction of a Rare-Cutter Physical Map from the 6
         Mb YAC Contig around D12S8
             Southern blots of total yeast plus YAC DNA,
10 digested to completion with rare-cutter enzymes (see
Materials and Methods) and separated on CHEF gels, were
hybridized sequentially with i) the STS used for the initial
screening of the YAC in question, ii) pYAC4 right arm
sequences, iii) pYAC4 left arm sequences, and iv) a human
15 ALU-repeat probe (BLUR-8). The long-range restriction map
that was obtained in this way, was completed by probing with
PCR-isolated STSs/YAC end probes. Occasionally double-
digests were performed.
             Restriction maps of individual YAC clones were
20 aligned and a consensus restriction map was established. It
is important to note here that the entire consensus rare-
cutter map was supported by at least two independent clones
showing a full internal consistency.

25 3.4. Physical mapping of CA repeats and monomorphic
         STSs/ESTs
             Based upon integrated mapping data as emerged from
the Second International Workshop on Human Chromosome 12
[44], a number of published markers was expected to be
30 mapping within the YAC contig presented here. To allow full
integration of our mapping data with those obtained by
others, a number of markers were STS content-mapped on our
contig, and the ones found positive were subsequently
sublocalized by (primer-)hybridization on YAC Southern
35 blots. Among the markers that were found to reside within
the contig presented here were CA repeats D12S313
(AFM207xf2) and D12S335 (AFM273vg9) [45], D12S375 (CHLC
GATA3F02), and D12S56 [46]. Furthermore, the interferon

gamma gene (IFNG) [47], the ras-related protein gene Rap1B
[48], and expressed sequence tag EST01096 [49] were mapped
using primer sets which we developed based on publicly
available sequence data (see Table II). Markers which were
5 tested and found negative included D12S80 (AFM102xd6),
D12S92 (AFM203va7), D12S329 (AFM249xh9) and D12S344
(AFM296xd9).

4. Discussion

10   In the present example the establishment of a YAC
contig and rare-cutter physical map covering approximately 6
Mb on 12q15, a region on the long arm of human chromosome 12
containing MAR in which a number of recurrent chromosomal
aberrations of benign solid tumors are known to be mapping
15 was illustrated.

The extent of overlap between individual YACs has
been carefully determined, placing the total length of the
contig at approximately 6 Mb (Fig. 1). It should be noted
that our sizing-data for some of the YAC clones differ
20 slightly from the sizes determined by CEPH [50]. It is our
belief that this is most probably due to differences in the
parameters for running the pulsed-field gels in the
different laboratories.

Using restriction mapping and STS-content
25 analysis, a consensus long range physical map (Fig. 1) was
constructed. The entire composite map is supported by at
least two-fold coverage. In total over 30 Mb of YAC DNA was
characterized by restriction and STS content analysis,
corresponding to an average contig coverage of about 5
30 times. Although the "inborn" limited resolution associated
with the technique of pulsed-field electrophoresis does not
allow very precise size estimations, comparison to
restriction mapping data obtained from a 500 kb cosmid
contig contained within the YAC contig presented here showed
35 a remarkable good correlation. Extrapolating from the cosmid
data, we estimate the accuracy of the rare-cutter physical
map presented here at about 10 kb.

The results of our physical mapping studies
allowed integration of three gene-specific as well as five
anonymous markers isolated by others (indicated in between
arrows in Fig. 1). The anonymous markers include one
5 monomorphic and four polymorphic markers. Five previously
published YAC-end-derived single copy STSs (RM1, RM4, RM5,
RM7, and RM21) as well as four published CA repeats (D12S56,
D12S313, D12S335, and D12S375) and three published gene-
associated STSs/ESTs (RAP1B, EST01096, and IFNG) have been
10 placed on the same physical map and this will facilitate
(linkage-) mapping and identification of a number of
traits/disease genes that map in the region. Furthermore, we
were able to place onto the same physical map, seventy two
YAC-end-derived (Table I) and eight cosmid-end- or inter-
15 ALU-derived DNA markers (CH9, RM1, RM110, RM111, RM130,
RM131, RM132, and RM133), which were developed during the
process of chromosome walking. The PYTHIA automatic mail
server at PYTHIA@anl.gov was used to screen the derived
sequences of these DNA markers for the presence of repeats.
20 For forty three of these seventy two DNA markers (listed in
Table II), primer sets were developed and the corresponding
STSs were determined to be single copy by PCR as well as
Southern blot analysis of human genomic DNA. The twenty nine
remaining DNA markers (depicted in the yellow boxes)
25 represent YAC-end-derived sequences for which we did not
develop primer sets. These YAC-end sequences are assumed to
be mapping to chromosome 12 on the basis of restriction
mapping. The final picture reveals an overall marker density
in this region of approximately one within every 70 kb.
30   The analysis of the contig presented here revealed
many CpG-rich regions, potentially HTF islands, which are
known to be frequently associated with housekeeping genes.
These CpG islands are most probably located at the 5' ends
of as yet unidentified genes: it has been shown that in 90%
35 of cases in which three or more rare-cutter restriction
sites coincide in YAC DNA there is an associated gene [51].
This is likely to be an underestimate of the number of genes
yet to be identified in this region because 60% of tissue-

specific genes are not associated with CpG islands [52] and
also because it is possible for two genes to be transcribed
in different orientations from a single island [53].

        While several of the YAC clones that were isolated
5 from the CEPH YAC library mark 1 were found to be chimeric,
overlapping YAC clones that appeared to be non-chimeric
based on FISH, restriction mapping and STS content analysis
could be obtained in each screening, which is in agreement
with the reported complexity of the library. The degree of
10 chimerism for the CEPH YAC library mark 1 was determined at
18% (12 out of 68) for the region under investigation here.
The small number of YACs from the CEPH YAC library mark 3
(only 7 MEGA YACs were included in this study) did not allow
a reliable estimation of the percentage of chimeric clones
15 present in this library. The average size of YACs derived
from the mark 1 library was calculated to be 381 kb ; non-
chimeric YACs (n=58) had an average size of 366 kb while
chimeric YACs (n=12) were found to have a considerable
larger average size; i.e. 454 kb.

20   In summary, we present a 6 Mb YAC contig
corresponding to a human chromosomal region which is
frequently rearranged in a variety of benign solid tumors.
The contig links over 84 loci, including 3 gene-associated
STSs. Moreover, by restriction mapping we have identified at
25 least 12 CpG islands which might be indicative for genes
residing there. Finally, four CA repeats have been
sublocalized within the contig. The integration of the
genetic, physical, and transcriptional maps of the region
provides a basic framework for further studies of this
30 region of chromosome 12. Initial studies are likely to focus
on MAR and ULCR12, as these regions contain the breakpoint
cluster regions of at least three distinct types of solid
tumors. The various YAC clones we describe here are valuable
resources for such studies. They should facilitate the
35 search for genes residing in this area and the
identification of those directly affected by the chromosome
12q aberrations of the various benign solid tumors.

23

EXAMPLE 2

1. Introduction

It was found that the 1.7 Mb Multiple Aberration Region on human chromosome 12q15 harbors recurrent
5 chromosome 12 breakpoints frequently found in different benign solid tumor types. In this example the identification of an HMG gene within MAR that appears to be of pathogenetical relevance is described. Using a positional cloning approach, the High Mobility Group protein gene HMGI-
10 C was identified within a 175 kb segment of MAR and its genomic organization characterized. By FISH, within this gene the majority of the breakpoints of seven different benign solid tumor types were pinpointed. By Southern blot and 3'-RACE analysis, consistent rearrangements in HMGI-C
15 and/or expression of altered HMGI-C transcripts were demonstrated. These results indicate a link between a member of the HMG gene family and benign solid tumor development.

20 2. Materials and methods

2.1. Cell culture and primary tumor specimens.

Tumor cell lines listed in Fig. 3 were established by a transfection procedure [54] and have been described before in [39, 24] and in an article of Van de Ven et al.,
25 Genes Chromosom. Cancer 12, 296-303 (1995) enclosed with this application as ANNEX 1. Cells were grown in TC199 medium supplemented with 20% fetal bovine serum and were assayed by standard cytogenetic techniques at regular intervals. The human hepatocellular carcinoma cell lines Hep
30 3B and Hep G2 were obtained from the ATCC (accession numbers ATCC HB 8064 and ATCC HB 8065) and cultured in DMEM/F12 supplemented with 4% Ultroser (Gibco/BRL). Primary solid tumors were obtained from various University Clinics.

35 2.2. YAC and cosmid clones

YAC clones were isolated from the CEPH mark 1 [57] and mark 3 [58] YAC libraries using a combination of PCR-based screening [59] and colony hybridization analysis.

Cosmid clones were isolated from an arrayed human chromosome
12-specific cosmid library (LL12NC01) [60] obtained from
Lawrence Livermore National Laboratory (courtesy P. de
Jong). LL12NC01-derived cosmid clones are indicated by their
5 microtiter plate addresses; i.e. for instance 27E12.

Cosmid DNA was extracted using standard techniques
involving purification over Qiagen tips (Diagen). Agarose
plugs containing high-molecular weight yeast + YAC DNA
(equivalent to 1×10^9 cells ml^{-1}) were prepared as described
10 before [61]. Plugs were thoroughly dialysed against four
changes of 25 ml $T_{10}E_1$ (pH 8.0) followed by two changes of
0.5 ml 1 x restriction buffer before they were subjected to
either pulsed-field restriction enzyme mapping or YAC-end
rescue. YAC-end rescue was performed using a vectorette-PCR
15 procedure in combination with direct solid phase DNA
sequencing, as described before in reference 61. Inter-Alu
PCR products were isolated using published oligonucleotides
TC65 or 517 [62] to which SalI-tails were added to
facilitate cloning. After sequence analysis, primer pairs
20 were developed using the OLIGO computer algorithm [61].

2.3. DNA labelling
DNA from YACs, cosmids, PCR products and
oligonucleotides was labelled using a variety of techniques.
25 For FISH, cosmid clones or inter-Alu PCR products of YACs
were biotinylated with biotin-11-dUTP (Boehringer) by nick
translation. For filter hybridizations, probes were radio-
labelled with $\alpha-^{32}P$-dCTP using random hexamers [62]. In case
of PCR-products smaller than 200 bp in size, a similar
30 protocol was applied, but specific oligonucleotides were
used to prime labelling reactions. Oligonucleotides were
labelled using $\gamma-^{32}P$-ATP.

2.4. Nucleotide sequence analysis and PCR amplification
35   Nucleotide sequences were determined as described
in Example 1. Sequencing results were analyzed using an
A.L.F. DNA sequencer™ (Pharmacia Biotech) on standard 30
cm, 6% Hydrolink^R, Long Range™ gels (AT Biochem). PCR

amplifications were carried out essentially as described
before [39].

2.5. Rapid amplification of cDNA ends (RACE)
5   Rapid amplification of 3' cDNA-ends (3'-RACE) was
performed using a slight modification of part of the
GIBCO/BRL 3'-ET protocol. For first strand cDNA synthesis,
adapter primer (AP2) AAG GAT CCG TCG ACA TC(T)$_{17}$ was used.
For both initial and secondary rounds of PCR, the universal
10 amplification primer (UAP2) CUA CUA CUA CUA AAG GAT CCG TCG
ACA TC was used as "reversed primer". In the first PCR round
the following specific "forward primers" were used: i) 5'-
CTT CAG CCC AGG GAC AAC-3' (exon 1), ii) 5'-CAA GAG GCA GAC
CTA GGA-3' (exon 3), or iii) 5'-AAC AAT GCA ACT TTT AAT TAC
15 TG-3' (3'-UTR). In the second PCR round the following
specific forward primers (nested primers as compared to
those used in the first round) were used: i) 5'-CAU CAU CAU
CAU CGC CTC AGA AGA GAG GAC-3' (exon 1), ii) 5'-CAU CAU CAU
CAU GTT CAG AAG AAG CCT GCT-3' (exon 4), or iii) 5'-CAU CAU
20 CAU CAU TTG ATC TGA TAA GCA AGA GTG GG-3' (3'-UTR). CUA/CAU-
tailing of the nested, specific primers allowed the use of
the directional CloneAmp cloning system (GIBCO/BRL).

3. Results
25 3.1. Development of cosmid contig and STS map of MAR segment
          During the course of a positional cloning effort
focusing on the long arm of human chromosome 12, we
constructed a yeast artificial chromosome (YAC) contig
spanning about 6 Mb and consisting of 75 overlapping YACs.
30 For a description thereof reference is made to Example 1.
This contig encompasses MAR (see also Fig. 2), in which most
of the chromosome 12q13-q15 breakpoints as present in a
variety of primary benign solid tumors (34 tumors of eight
different types tested sofar; Table 5) and tumor cell lines
35 (26 tested sofar, derived from lipoma, uterine leiomyoma,
and pleomorphic salivary gland adenoma; Fig. 3) appear to
cluster. We have developed both a long-range STS and rare
cutter physical map of MAR and found, by FISH analysis, most

26

of the breakpoints mapping within the 445 kb subregion of
MAR located between STSs RM33 and RM98 (see Fig. 2 and 3).
FISH experiments, including extensive quality control, were
performed according to routine procedures as described
5   before [25, 39, 24, 42, 36] To further refine the
distribution of breakpoints within this 445 kb MAR segment,
a cosmid contig consisting of 54 overlapping cosmid clones
has been developed and a dense STS map (Fig. 2) established.
The cosmid contig was double-checked by comparison to the
10 rare cutter physical map and by STS content mapping.

3.2. Clustering of the chromosome 12q breakpoints within a
175 kb DNA segment of MAR
The chromosome 12q breakpoints in the various
15 tumor cell lines studied was pinpointed within the cosmid
contig by FISH (Fig. 3). As part of our quality control FISH
experiments [25, 39, 24, 42], selected cosmids were first
tested on metaphase spreads derived from normal lymphocytes.
FISH results indicated that the majority (at least 18 out of
20 the 26 cases) of the chromosome 12 breakpoints in these
tumor cell lines were found to be clustering within the 175
kb DNA interval between RM99 and RM133, indicating this
interval to constitute the main breakpoint cluster region.
FISH results obtained with Li-501/SV40 indicated that part
25 of MAR was translocated to an apparently normal chromosome
3; a chromosome aberration overseen by applied cytogenetics.
Of interest to note, finally, is the fact that the
breakpoints of uterine leiomyoma cell lines LM-5.1/SV40, LM-
65/SV40, and LM-608/SV40 were found to be mapping within the
30 same cosmid clone; i.e. cosmid 27E12.
We also performed FISH experiments on eight
different types of primary benign solid tumors with
chromosome 12q13-q15 aberrations (Table 4). A mixture of
cosmid clones 27E12 and 142H1 was used as molecular probe.
35 In summary, the results of the FISH studies of primary
tumors were consistent with those obtained for the tumor
cell lines. The observation that breakpoints of each of the
seven different tumor types tested were found within the

same 175 kb DNA interval of MAR suggested that this interval
is critically relevant to the development of these tumors
and, therefore, might harbor the putative MAG locus or
critical part(s) of it.

5

3.3. Identification of candidate genes mapping within MAR
        In an attempt to identify candidate genes mapping
within the 175 kb subregion of MAR between STSs RM99 and
RM133, we used 3'-terminal exon trapping and genomic
10 sequence sampling (GSS) [63]. Using the GSS approach, we
obtained DNA sequence data of the termini of a 4.9 kb BamHI
subfragment of cosmid 27E12, which was shown by FISH
analysis to be split by the chromosome 12 aberrations in
three of the uterine leiomyoma cell lines tested. A BLAST
15 [64] search revealed that part of these sequences displayed
sequence identity with a publicly available partial cDNA
sequence (EMBL accession #. Z31595) of the high mobility
group (HMG) protein gene HMGI-C [65], which is a member of
the HMG gene family [66]. In light of these observations,
20 HMGI-C was considered a candidate MAG gene and studied in
further detail.

3.4. Genomic organization of HMGI-C and rearrangements in
     benign solid tumors
25   Since only 1200 nucleotides of the HMGI-C
transcript (reported size approximately 4 kb [65, 67]) were
publicly available, we first determined most of the
remaining nucleotide sequences of the HMGI-C transcript
(GenBank, # U28749). This allowed us to subsequently
30 establish the genomic organization of the gene. Of interest
to note about the sequence data is that a CT-repeat is
present in the 5'-UTR of HMGI-C and a GGGGT-pentanucleotide
repeat in the 3'-UTR, which might be of regulatory
relevance. Comparison of transcribed to genomic DNA
35 sequences (GenBank, # U28750, U28751, U28752, U28753, and
U28754) of the gene revealed that HMGI-C contains at least 5
exons (Fig. 2). Transcriptional orientation of the gene is
directed towards the telomere of the long arm of the

chromosome. Each of the first three exons encode a putative
DNA binding domain (DBD), and exon 5 encodes an acidic
domain, which is separated from the three DBDs by a spacer
domain encoded by exon 4. The three DBD-encoding exons are
5 positioned relatively close together and are separated by a
large intron of about 140 kb from the two other exons, which
in turn are separated about 11 kb from each other. Of
particular interest to emphasize here is that the five exons
are dispersed over a genomic region of at least 160 kb, thus
10 almost covering the entire 175 kb main MAR breakpoint
cluster region described above. Results of molecular
cytogenetic studies, using a mixture of cosmids 142H1
(containing exons 1-3) and 27E12 (containing exons 4 and 5)
as molecular probe, clearly demonstrate that the HMGI-C gene
15 is directly affected by the observed chromosome 12
aberrations in the majority of the tumors and tumor cell
lines that were evaluated (Fig. 3; Table 4). These
cytogenetic observations were independently confirmed by
Southern blot analysis in the case of LM-608/SV40 (results
20 not shown) LM-30.1/SV40 [24], and Ad-312/SV40; probes used
included CH76, RM118-A, and EM26. The failure to detect the
breakpoints of LM-65/SV40, LM-609/SV40, Ad-211/SV40, Ad-
263/SV40, Ad-302/SV40, Li-14/SV40, and Li-538/SV40 with any
of these three probes was also consistent with the FISH data
25 establishing the relative positions of the breakpoints in
MAR (cf. Fig. 3). These results made HMGI-C a prime
candidate to be the postulated MAG gene.

3.5. Expression of aberrant HMGI-C transcripts in benign
30 solid tumor cells.
         In the context of follow-up studies, it was of
interest to test for possible aberrant HMGI-C expression.
Initial Northern blot studies revealed that transcripts of
HMGI-C could not be detected in a variety of normal tissues
35 (brain, heart, lung, liver, kidney, pancreas, placenta,
skeletal muscle) tested as well as in a number of the tumor
cell lines listed in Fig. 3 (data not shown). It is known
that HMGI-C mRNA levels in normal differentiated tissues are

very much lower than in malignant tissues [65, 67]. As a
control in our Northern studies, we included hepatoma cell
line Hep 3B, which is known to express relatively high
levels of HMGI-C. We readily detected two major HMGI-C

5 transcripts, approximately 3.6 and 3.2 kb in size; with the
differences in molecular weight most likely due to
differences in their 5'-noncoding regions. In an alternative
and more sensitive approach to detect HMGI-C or 3'-aberrant
HMGI-C transcripts, we have performed 3'-RACE experiments.

10 In control experiments using a number of tissues with
varying HMGI-C transcription levels (high levels in Hep 3B
hepatoma cells, intermediate in Hep G2 hepatoma cells, and
low in myometrium, normal fat tissue, and pseudomyxoma), we
obtained 3'-RACE clones which, upon molecular cloning and

15 nucleotide sequence analysis, appeared to represent perfect
partial cDNA copies of 3'-HMGI-C mRNA sequences; no matter
which of the three selected primer sets was used (see
Methodology). RACE products most likely corresponding to
cryptic or aberrantly spliced HMGI-C transcripts were

20 occasionally observed; their ectopic sequences were mapped
back to HMGI-C intron 3 or 4.

            In similar 3'-RACE analysis of ten different
primary tumors or tumor cell lines derived from lipoma,
uterine leiomyoma, and pleomorphic salivary gland adenoma,

25 we detected both constant and unique PCR products. The
constant PCR products appeared to represent, in most cases,
perfect partial cDNA copies of 3'-HMGI-C mRNA sequences.
They most likely originated from a presumably unaffected
HMGI-C allele and might be considered as internal controls.

30 The unique PCR products of the ten tumor cell samples
presented here appeared to contain ectopic sequences fused
to HMGI-C sequences. In most cases, the ectopic sequences
were found to be derived from the established translocation
partners, thus providing independent evidence for

35 translocation-induced rearrangements of the HMGI-C gene.
Information concerning nucleotide sequences, diversion
points, and chromosomal origins of the ectopic sequences of
these RACE products is summarized in Table 5. It should be

30

noted that chromosomal origins of ectopic sequences was
established by CASH (Chromosome Assignment using Somatic
cell Hybrids) analysis using the NIGMS Human/Rodent Somatic
Hybrid Mapping Panel 2 obtained from the Coriell Cell
5 Repositories. Chromosomal assignment was independently
confirmed by additional data for cases pCH1111, pCH172,
pCH174, pCH193, and pCH117, as further outlined in Table 5.
Taking into account the limitations of conventional
cytogenetic analysis, especially in cases with complex
10 karyotypes, the chromosome assignments of the ectopic
sequences are in good agreement with the previous
cytogenetic description of the translocations.

        Somewhat unexpected were the data obtained with
Ad-312/SV40, as available molecular cytogenetic analysis had
15 indicated its chromosome 12 breakpoint to map far outside
the HMGI-C gene; over 1 Mb [42]. The ectopic sequences
appeared to originate from chromosome 1 (more precisely from
a segment within M.I.T. YAC contig WC-511, which is
partially mapping at 1p22), the established translocation
20 partner (Fig. 2). Further molecular analysis is required to
precisely define the effect on functional expression of the
aberrant HMGI-C gene in this particular case. Of further
interest to note here, is that the sequences coming from
chromosome 1 apparently remove the GGGGT repeat observed in
25 the 3'-UTR region of HMGI-C, as this repeat is not present
in the RACE product. In contrast, in primary uterine
leiomyoma LM-#58 (t(8;12)(q24;q14-q15)), which was shown to
have its breakpoint also in the 3'-UTR, this repeat appeared
to be present in the RACE product. Therefore, removal of
30 this repeat is most probably not critical for tumor
development. The results with primary tumor LM-#168.1, in
which the X chromosome is the cytogenetically assigned
translocation partner, revealed that the ectopic sequences
were derived from chromosome 14; the preferential
35 translocation partner in leiomyoma. It is possible that
involvement of chromosome 14 cannot be detected by standard
karyotyping in this particular case, as turned out to be the
case for Li-501/SV40. In primary lipoma Li-#294

(t(8;12)(q22;q14)), two alternative ectopic sequences were
detected. Additional CASH analysis using a hybrid cell
mapping panel for regional localization of probes to human
chromosome 8 [68] revealed that these were both derived from
5 chromosome 8q22-qter (Table 5). It is very well possible
that these RACE products correspond to alternatively spliced
transcripts. Finally, in four of the cases (Table 5, cases
pCH114, pCH110, pCH109, pCH116), the RACE products appeared
to correspond to cryptic or aberrantly spliced HMGI-C
10 transcripts, as the corresponding ectopic sequences were
found to be derived from either HMGI-C intron 3 or 4. Such
RACE products have also been observed in the control
experiments described above. In conclusion, the detection of
aberrant HMGI-C transcripts in the tumor cells provides
15 additional strong support of HMGI-C being consistently
rearranged by the various chromosome 12 aberrations. It
should be noted that the aberrant HMGI-C transcripts in the
various cases should be characterized in full length before
any final conclusion can be drawn about biological
20 implications.

        A first and preliminary evaluation of isolated
ectopic sequences revealed in phase open reading frames of
variable length. In the case of primary tumor LM-#25, for
instance, already the second codon in the ectopic sequences
25 appeared to be a stop codon (Table 5). A note of caution is
appropriate here, as sequence data have been obtained only
for clones that were produced via two rounds of extensive
(probably mutations inducing) PCR. For Li-501/SV40, it is of
interest to note that, in Northern blot analysis, the
30 isolated ectopic sequences detected a transcript of over 10
kb in a variety of tissues, including heart, kidney, liver,
lung, pancreas, placenta, and skeletal muscle, but not in
brain (data not shown). As chromosome 3 is the preferred
partner in the chromosome 12q13-q15 translocations in
35 lipomas and the chromosome 3 breakpoints of various lipomas
were found to be spanned by YAC clone CEPH192B10, the
detected transcript might correspond to a putative lipoma-
preferred partner gene (LPP).

4. Discussion

          In ANNEX 1 it was demonstrated that the chromosome
12q13-q15 breakpoints of lipoma, pleomorphic salivary gland
adenoma, and uterine leiomyoma, irrespective of their
5 cytogenetic assignments in the past to segment q13, q14, or
q15 of chromosome 12, all cluster within the 1.7 Mb DNA
interval designated MAR. In support of the claimed
clustering of breakpoints is a recent study by Schoenberg
Fejzo et al. [14], identifying a CEPH mega-YAC spanning the
10 chromosome 12 translocation breakpoints in two of the three
tumor types. In the present study, we have conclusively
demonstrated by FISH analysis that chromosome 12 breakpoints
of seven different solid tumor types are clustering within a
relatively small (175 kb) segment of MAR. For some tumor
15 cell lines, Southern blot data were obtained and these were
always in support of the FISH results. From all these
observations, we conclude that this segment of MAR
constitutes a major target area for the chromosome 12
aberrations in these tumors and that it is likely to
20 represent the postulated MAG locus: the multi-tumor aberrant
growth locus that might be considered as common denominator
in these tumors.

          Within the 175 kb MAR segment, we have identified
the HMGI-C gene and determined characteristics of its
25 genomic organization. Structurally, the HMGI-C-encoded
phosphoprotein consists of three putative DNA binding
domains, a spacer region, and an acidic carboxy-terminal
domain, and contains potential sites of phosphorylation for
both casein kinase II and p34/cdc2 [65, 67]. We have
30 provided strong evidence that HMGI-C is a prime candidate
target gene involved in the various tumor types studied
here. In FISH studies, the breakpoints of 29 out of 33
primary tumors were found to be mapping between two highly
informative cosmids 142H1 and 27E12; the first one
35 containing the three DBD-encoding exons and the second one,
the remaining exons that code for the two other domains.
Therefore, the majority of the breakpoints map within the
gene, most of them probably within the 140 kb intron (intron

3), which is also in line with FISH results obtained with
the 26 tumor cell lines that were evaluated. It should also
be noted that the 5'-end of the HMGI-C gene is not yet fully
characterized. As HMGI(Y), another member of this gene
5 family, is known to possess various alternative first exons
[69], the size of the HMGI-C gene might be larger than yet
assumed. Further support that HMGI-C is affected by the
chromosome 12 aberrations can be deduced from the results of
the 3'-RACE experiments. Aberrant HMGI-C transcripts were
10 detected in tumor cells, consisting of transcribed HMGI-C
sequences fused to newly acquired sequences, in most cases
clearly originating from the chromosomes that were
cytogenetically identified as the translocation partners. It
is noteworthy that many chromosomes have been found as
15 translocation partner in the tumors studied. This observed
heterogeneity in the reciprocal breakpoint regions involved
in these translocations resembles that of a variety of
hematological malignancies with chromosome 11q23
rearrangements involving the MLL gene [70], the
20 translational product of which carries an amino-terminal
motif related to the DNA-binding motifs of HMGI proteins.
        An intriguing issue pertains to the effect of the
chromosome 12 aberrations on expression of the HMGI-C gene
and the direct physiological implications. Some functional
25 characteristics of HMGI-C are known or may be deduced
speculatively from studies of other family members. As it
binds in the minor groove of DNA, it has been suggested that
HMGI-C may play a role in organising satellite chromatin or
act as a transcription factor [71, 72]. Studies on HMGI(Y),
30 which is the member most closely related to HMGI-C, have
suggested that HMGI(Y) may function as a promoter-specific
accessory factor for NF-κ B transcriptional activity [73].
HMGI(Y) has also been shown to stimulate or inhibit DNA
binding of distinct transcriptional factor ATF-2 isoforms
35 [74]. Both studies indicate that the protein may simply
constitute a structural component of the transcriptional
apparatus functioning in promoter/enhancer contexts. In a
recent report on high mobility group protein 1 (HMG1), yet

34

another member of the HMG gene family with a similar domain
structure as HMGI-C and acting as a quasi-transcription
factor in gene transcription, a truncated HMG1 protein
lacking the acidic carboxy-terminal region was shown to
5   inhibit gene transcription [75]. It was put forward that the
acidic terminus of the HMG1 molecule is essential for the
enhancement of gene expression in addition to elimination of
the repression caused by the DNA binding. As most of the
chromosome 12 breakpoints seem to occur in the 140 kb
10   intron, separation of the DBDs from the acidic carboxy-
terminal domain seems to occur frequently. In case the
acidic domain in HMGI-C has a similar function as the one in
HGMI(Y), the result of the chromosome 12 aberrations is
likely to affect gene expression. Finally, it should be
15   noted that the fate of the sequences encoding the acidic
carboxy-terminal region is not yet known.

          As HMGI-C is the first member of the HMG gene
family that might be implicated in the development of benign
tumors, the question arises as to whether other members of
20   this family could also be involved. The HMG protein family
consists of three subfamilies: i) the HMG1 and 2 type
proteins, which have been found to enhance transcription in
vitro and may well be members of a much larger class of
regulators with HMG boxes; ii) the random-coil proteins
25   HMG14 and 17 with an as yet unknown function; iii) the HMGI-
type proteins, which bind to the minor groove and include
HMGI-C, HMGI, and HMGI-Y; the latter two are encoded by the
same gene. It is of interest to note that published mapping
positions of members of the HMG family coincide with
30   published chromosome breakpoints of benign solid tumors such
as those studied here. The HMGI(Y) gene, for instance, has
been mapped to human chromosome 6p21 [69], which is known to
be involved in recurrent translocations observed in uterine
leiomyoma, lipoma, and pleomorphic salivary gland adenoma
35   [76]. As listed in the Human Genome Data Base, not all known
members of the HMG family have been chromosomally assigned
yet, although for some of them a relatively precise mapping
position has been established. For instance, HMG17 to

chromosome 1p36.1-p35, HMG1L to 13q12, and HMG14 to 21q22.3;
all chromosome segments in which chromosome breakpoints of
the tumor types studied here have been reported [76].
Whether HMGI(Y) or any other of these HMG members are indeed
5 affected in other subgroups of these tumors remains to be
established. Of interest to mention, furthermore, are
syndromes such as Bannayan-Zonana (McKusick #153480),
Proteus (McKusick #176920), and Cowden (McKusick #158350);
the latter syndrome is also called multiple hamartoma
10 syndrome. In 60% of the individuals with congenital
Bannayan-Zonana syndrome, a familial macrocephaly with
mesodermal hamartomas, discrete lipomas and hemangiomas were
found [70].

        Finally, one aspect of our results should not
15 escape attention. All the tumors that were evaluated in this
study were of mesenchymal origin or contained mesenchymal
components. It would be of great interest to find out
whether the observed involvement of HMGI-C is mesenchyme-
specific or may be found also in tumors of non-mesenchymal
20 origin. The various DNA clones we describe here are valuable
resources to address this important issue and should
facilitate studies to conclusively implicate the HMGI-C gene
in tumorigenesis.

25 **EXAMPLE 3**
Rearrangements of another member of the HMG gene family

1. Introduction
        This example clearly demonstrates that within a
30 given tumor entity (e.g. pulmonary chondroid hamartomas,
uterine leiomyomas, endometrial polyps) tumors,
histologically practically indistinguishable from each
other, arise if either the HMGI-C gene or the HMGI(Y) gene
is affected by chromosomal rearrangements. Thus, indeed a
35 group of genes leading to aberrant mesenchymal growth
including but not restricted to HMGI-C and HMGI(Y) can be
defined.

36

2. Material and methods

2.1. Chromosome preparation

Chromosome preparation followed routine methods. Cells were treated with 30 μl colcemide (10μg/ml) for 2-3 h and then harvested using the trypsin method (0.05% trypsin, 0.02% EDTA) followed by a hypotonic shock in six fold diluted medium TC 199 for 20 minutes at room temperature and methanol:acetic acid (3:1) fixation. Chromosomes were then GTG-banded.

2.2. In situ hybridization

In situ hybridisation was performed as outlined in one of the previous examples.

2.3. PAC library screening

The PAC library (Genome Systems Library Screening Service, St. Louis, Missouri, USA) was screened by PCR with a primer set specific for the HMGI(Y) gene. For screening we designed the forward primer with the sequence:

5'-CTC CAA GAC AGG CCT CTG ATG T-3' (intron 3)

and the reverse primer:

5'-ACC ACA GGT CCC CTT CAA ACT A-3' (intron 3)

giving rise to a fragment of 338 bp. For amplification the following thermal cycling was used: 94°C, 5 min, (94°C, 1 min, 59°C, 1 min, 72°C, 2 min) x 30, 72°C, 10 min.

2.4. DNA preparations from PAC clones

Bacterial colonies containing single PAC clones were inoculated into LB medium and grown overnight at 37°C. 660 μl of the overnight culture were diluted into 25 ml of LB medium and grown to an OD_{550} of 0.05-0.1. By addition of IPTG to a final concentration of 0.5 mM the P1 lytic replicon was induced. After addition of IPTG, growth was continued to an OD_{550} of 0.5-1.5, and plasmid DNA was extracted using the alkaline lysis procedure recommended by Genome Systems.

3. Results

The primer set for screening the human PAC library
was designed from sequences belonging to intron 3 of
HMGI(Y). Because of sequence homology between HMGI-C and
5   HMGI(Y) the amplified sequence of 338 bp was tested by
homology search to be specific exclusively for HMGI(Y).
Library screening resulted in three positive PAC clones that
had an average insert length of approximately 100 kb. Two of
these clones (Pac604, Pac605) were used for the following
10 FISH studies. In order to prove if HMGI(Y) is rearranged in
tumors with translocations involving 6p21.3 in either simple
or complex form we performed FISH analysis on metaphase
spreads from four primary pulmonary chondroid hamartomas and
two endometrial polyps all with 6p21.3 abnormalities.
15 For each case 20 metaphases were scored. At least one of the
two PAC clones Pac604 and Pac605 described above was across
the breakpoint in all six cases analyzed. These results
clearly show that the breakpoints of the tumors with 6p21
aberrations investigated herein are clustered either within
20 the HMGI(Y) gene or its close vicinity.

EXAMPLE 4
Hybrid HMGI-C in lipoma cells.
cDNA clones of the chromosome 3-derived lipoma-
25 preferred partner gene LPP (>50 kb) were isolated and the
nucleotide sequence thereof established. Data of a composite
cDNA are shown in Fig. 4. An open reading frame for a
protein (612 amino acids (aa)) with amino acid sequence
similarity (over 50%) to zyxin of chicken was identified.
30 Zyxin is a member of the LIM protein family, whose members
all possess so-called LIM domains [78]. LIM domains are
cysteine-rich, zinc-binding protein sequences that are found
in a growing number of proteins with divers functions,
including transcription regulators, proto-oncogene products,
35 and adhesion plaque constituents. Many of the LIM family
members have been postulated to play a role in cell
signalling and control of cell fate during development.
Recently, it was demonstrated that LIM domains are modular

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protein-binding interfaces [79]. Like zyxin, which is
present at sites of cell adhesion to the extracellular
matrix and to other cells, the deduced LPP-encoded protein
(Fig. 6) possesses three LIM domains and lacks classical
5 DNA-binding homeodomains.

In 3'-RACE analysis of Li-501/SV40, a HMG1-C
containing fusion transcript was identified from which a
hybrid protein (324 aa) could be predicted and which was
subsequently predicted to consist of the three DBDs (83 aa)
10 of HMG1-C and, carboxy-terminally of these, the three LIM
domains (241 aa) encoded by LPP. In PCR analysis using
approriate nested amplimer sets similar HMGI-C/LPP hybrid
transcripts were detected in various primary lipomas and
lipoma cell lines carrying a t(3;12) and also in a
15 cytogenetically normal lipoma. These data reveal that the
cytogenetically detectable and also the hidden t(3;12)
translocations in lipomas seem to result consistently in the
in-phase fusion of the DNA-binding molecules of HMG1-C to
the presumptive modular protein-binding interfaces of the
20 LPP-encoded protein, thereby replacing the acidic domain of
HMG1-C by LIM domains. Consequently, these protein-binding
interfaces are most likely presented in the nuclear
environment of these lipoma cells, where they might affect
gene expression, possibly leading to aberrant growth
25 control. Out of the large variety of benign mesenchymal
tumors with chromosome 12q13-q15 aberrations, this is the
first example of a chromosome translocation partner
contributing recurrently and consistently to the formation
of a well-defined tumor-associated HMG1-C fusion protein.
30   Figure 5 shows the cDNA sequence of the complete
isolated LPP gene.

EXAMPLE 5

Diagnostic test for lipoma
35   A biopsy of a patient having a lipoma was taken.
From the material thus obtained total RNA was extracted
using the standard TRIZOL™ LS protocol from GIBCO/BRL as
described in the manual of the manufacturer. This total RNA

was used to prepare the first strand of cDNA using reverse
transcriptase (GIBCO/BRL) and an oligo dT(17) primer
containing an attached short additional nucleotide stretch.
The sequence of the primer used is as described in Example
5 2, under point 2.5.. RNase H was subsequently used to
remove the RNA from the synthesized DNA/RNA hybrid molecule.
PCR was performed using a gene specific primer (Example 2,
point 2.5.) and a primer complementary to the attached short
additional nucleotide stretch. The thus obtained PCR product
10 was analysed by gel electroforesis. Fusion constructs were
detected by comparing them with the background bands of
normal cells of the same individual.

      In an additional experiment a second round of
hemi-nested PCR was performed using one internal primer and
15 the primer complementary to the short nucleotide stretch.
The sensitivity of the test was thus significantly improved.
      Figure 8 shows a typical gel.

EXAMPLE 6
20 Aberrations of 12q14-15 and 6p21 in pulmonary chondroid
hamartomas
<u>1. Introduction</u>
      Pulmonary chondroid hamartomas (PCH) are often
detected during X-ray examination of the lung as so-called
25 coin lesions. However, lung metastases of malignant tumors
and rarely lung cancers can also present as coin lesions.
This example shows that FISH requiring a minimal amount of
tumor cells can be used to correctly distinguish between the
majority of PCHs and malignant tumors. Thus the test can
30 successfully be applied e.g. to tumor cells obtained by fine
needle aspiration.

<u>2. Materials and methods</u>
      Samples from a total of 80 histologically
35 characterized PCHs were included in this study.
Cell cultures, chromosome preparations and FISH were
obtained or performed as described in the previous examples.

40

<u>3. Results</u>

Cytogenetic studies revealed that of the 80 PCHs studied cytogenetically 51 revealed detectable aberrations involving either 12q14-15 or 6p21. By FISH using either a
5 pool of cosmids belonging to the HMGI-C gene or using the PAC clones of HMGI(Y) described in the previous example we were able to detect hidden structural rearrangements of those regions in 4 additional cases (3 with 12q and one with 6p involvement). Therefore, the FISH test alone can be used
10 for a kit to precisely detect the rearrangement of either the HMGI-C or the HMGI(Y) gene rearrangements in more than 50% of the PCHs and is thus a valuable additonal tool for the diagnosis of these tumors (without being restricted to this type of tumors as shown in two of the other examples).
15

EXAMPLE 7
Diagnosis of soft tissue tumors particularly of adipocytic origin
<u>1. Introduction</u>
20   Adipocyte tissue tumors often cause diagnostic difficulties particularly when material taken from fine needle aspiration biopsies or cryosections has to be evaluated. This examples demonstrates the validity of the FISH test for the differential diagnosis of adipocyte tissue
25 tumors and rare soft tissue tumors.

<u>2. Materials and methods</u>
2.1. Tumor samples
        Tumor samples from three soft tissue tumors were
30 investigated by FISH. Sample one (1) was from a adipocytic tumor and histologically it was either an atypical lipoma or a well-differentiated liposarcoma. The second case (tumor 2) was diagnosed to be most likely a myxoid liposarcoma but other types of malignant soft tissue tumors including
35 aggressive angiomyxoma were also considered. The third tumor (tumor 3) was also of adipocytic origin and both a lipoma and a well differentiated liposarcoma were considered.

2.2. Isolation of cells and FISH

The tumor samples were enzymatically disaggregated
following routine methods. The resulting single cell
suspensions were centrifuged and the suspensions were fixed
5 using methanol:glacial acetic acid (3:1) at room temperature
for 1 hour. The cell suspensions were then dropped on clean
dry slides and allowed to age for 6 hours at 60°C. FISH was
performed using molecular probes from the HMGI-C gene as
described in the previous examples.
10

3. Results

At the interphase level tumor 1 and 2 both showed
split signals for one of the alleles. These findings are
compatible with the diagnosis of benign tumors i.e. an
15 atypical lipoma in the first case and an aggressive
angiomyxoma in the second case. They allowed to rule out the
presence of malignant adipocytic tissue tumors.

In the third case the FISH revealed a high degree
of amplification of the MAR region or part of it. Since the
20 amplification units observed in giant marker or ring
chromosomes in well-differentiated liposarcomas can involve
the MAR region these findings leads to the diagnosis of a
well-differentiated liposarcoma. The three cases presented
within this example show the usefulness of the DNA probes
25 described. They can be used in a kit for a relatively simple
and fast interphase FISH experiment offering an additional
tool for the diagnosis of soft tissue tumors.

EXAMPLE 8
30 Expression of the HMGI-C gene in normal tissue
1. Introduction

It is the aim of this example to show that the
expression of the HMGI-C gene is mainly restricted to human
tissues during embryonic and fetal development. In contrast,
35 in most normal tissues of the adult particularly, including
those tissues and organs tumors with HMGI-C rearrangements
can arise from, no expression can be noted. This indicates
that even the transcriptional re-activation of the gene can

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inititate tumorigenesis. On the other hand it underlines the
usefulness of antisense strategies (including those
antisense molecules directed towards the normal HMGI-C mRNA)
to inhibit or stop tumor growth.

5

2. Materials and methods
2.1. Tissue samples
        All adult tissue samples used for this study were
taken from surgically removed tissue frozen in liquid
10 nitrogen within a period of 15 min after removal. Most of
the samples were from adjacent normal tissue removed during
tumor surgery. In detail we have used 8 samples taken from
fat tissues at various anatomical sites, 20 samples taken
from myometrial tissue, 8 samples taken from lung tissue, 4
15 samples taken from the salivary glands (Glandula parotis and
Glandula submandibularis), one tissue sample taken from the
heart muscle, 25 samples taken from breast tissue from
patients of different ages, 2 samples from the brain, 3
liver samples, 7 samples taken from renal tissue, and
20 embryonic/fetal tissue (extremeties, 6 samples) from
embryos/fetuses (10-14th gestational week) after abortion
from socio-economic reasons.
        In addition, three cell lines were used: As a
control for HMGI-C expression we used the hepatoma cell line
25 Hep 3B and the cell line L14 established from a lipoma with
the typical translocation t(3;12). HeLa cells were used as a
negative control because RT experiments reproduced for 10
times did not reveal HMGI-C expression in our own studies.

30 2.2. RT-PCR for the expression of HMGI-C
        100 mg of tissue sample was homogenized, and RNA
was isolated using the trizol reagent (GibcoBRL, Eggenstein,
Germany) containing phenol and isothiocyanate. cDNA was
synthesized using a poly(A)-oligo(dt)17 primer and M-MLV
35 reverse transcriptase (GibcoBRL, Eggenstein, Germany). Then,
a hemi-nested PCR was performed.
        For first and second PCR the same lower primer
(Revex 4) (5'-TCC TCC TGA GCA GGC TTC-3' (exon 4/5)) was

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used. In the first round of PCR the specific upper primer
(SE1) (5'-CTT CAG CCC AGG GAC AAC-3' (exon 1)), and in the
second round of PCR the nested upper primer (P1) (5'-CGC CTC
AGA AGA GAG GAC-3' (exon 1)) was used. Both rounds of PCR
5 were performed in a 100 μl volume containing 10 mM Tris/HCl
pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatin, 100 μM
dATP, 100 μM dTTP, 100 μM dGTP, 100 μM dCTP, 200 nM upper
primer, 200 nM lower primer, and 1 unit/100 μl AmpliTaq
polymerase (Perkin Elmer, Weiterstadt, Germany).
10 Amplification was performed for 30 cycles (1 min 94°C, 1 min
53°C, 2 min 72°C). As template in the first round of PCR
cDNA derived from 250 ng total RNA, and in the second round
of PCR 1 μl of the first PCR reaction mix was used.

15 2.3. Control assay for intact mRNA/cDNA
          As control reaction for intact RNA and cDNA PCR a
test based on the amplification of the cDNA of the
housekeeping gene glyceraldehyde 3-phosphate dehydrogenase
(GAPDH). PCR reaction was performed for 35 cycles under the
20 same conditions as described above for the first round of
PCR of HMGI-C expression.

3. Results
          As for the expression studies all experiments were
25 repeated at least twice. To assure that all RNA and cDNA
preparations used for the RT-PCRs were intact (otherwise
resulting in false negative results) routinely a RT-PCR for
expression of the housekeeping gene GAPDH was performed. A
positive GAPDH RT-PCR results in a 299 bp fragment. Only
30 samples revealing a positive GAPDH RT-PCR were included in
this study. As the result of RT-PCR in HMGI-C positive cells
such as Hep 3B and L14 a specific 220 bp fragment is
detectable. HeLa cells did not show an expression of HMGI-C.
Except for two myometrial samples (most likely due to myomas
35 at a submicroscopic level) all normal tissue samples taken
from adult individuals did not show any detectable level of
HMGI-C expression. In contrast, all fetal/embryonic tissues
tested revealed HMGI-C expression.

44

EXAMPLE 9

Expression of the HMGI-C gene as a diagnostic tool for the
early detection of leukemias

1. Introduction

5   Cytogenetically detectable aberrations affecting
the HMGI-C gene have been found in a variety of benign solid
tumors of mesenchymal origin. Apparently, the aberrations
also lead to the transcriptional activation of the gene.
Since blood cells are also of mesenchymal origin, it was
10 tempting to check leukemic cells for HMGI-C expression. The
present example shows that the activation of the gene in
cells of the peripheral blood is a suitable marker
indicating immature cells/abnormal stem cells found in
leukemias. Since the expression of HMGI-C can be determined
15 with a high degree of sensitivity the RT-PCR for the
expression of the gene can be used for a very early
detection of various hematological diseases.

2. Materials and methods

20   Samples from peripheral blood of 27 patients with
different types of leukemias including 19 patients with
Philadelphia-chromosome positive CML, 5 patients with AML,
and 3 patients with ALL were used for determination of HMGI-
C expression. Blood samples from 15 healthy probands served
25 as controls.
           RT-PCR for the expression of HMGI-C was performed
as outlined in example 8.

3. Results

30   Whereas expression of HMGI-C was clearly
detectable in all blood samples from leukemic patients there
was no expression noted in any of the blood samples taken
from the control persons. There is no evidence that the
transcriptional activation of the gene is due to mutations
35 affecting the gene or its surroundings. It is more
reasonable to assume that the activation is rather a
secondary effect related to the immaturity of the cells or
their abnormal proliferation. However, the high and even

improvable sensitivity makes e.g. a kit based on the RT-PCR
for the expression of the HMGI-C gene a very suitable
diagnostic tool.

5 **EXAMPLE 10**
The transcriptional re-expression of the HMGI-C gene can
lead to the initiation of the tumors
1. Introduction
            This example clearly shows that for some tumor
10 entities chromosomal breakpoints located 5' of the HMGI-C
gene do also exist indicating that the transcriptional up-
regulation of the gene is sufficient to initiate growth of
the corresponding tumor types.

15 2. Materials and methods
    2.1. Cell Culture
            After surgery the tumor samples (three pulmonary
chondroid hamartomas, one uterine leiomyoma) were washed
with Hank's solution supplemented with penicillin (200
20 IU/ml) and streptomycin (200 μg/ml). Tumors were
disaggregated with collagenase for 5-6 h at 37°C. The
suspension containing small fragments and single cells was
resuspended in culture medium TC 199 with Earle's salts
supplemented with 20% fetal bovine serum, 200 IU/ml
25 penicillin, and 200 μg/ml streptomycin.

    2.2. Chromosome preparations
            Chromosome preparation followed routine methods.
Cells were treated with 30 μl colcemide (10μg/ml) for 2-3 h
30 and then harvested using the trypsin method (0.05% trypsin,
0.02% EDTA) followed by a hypotonic shock in six fold
diluted medium TC 199 for 20 minutes at room temperature and
methanol:acetic acid (3:1) fixation. Chromosomes were then
GTG-banded.
35
    2.3. FISH studies
            To identify the chromosomes unambiguously, FISH
was performed after GTG-banding of the same metaphase

spreads. As DNA probes we used five cosmids belonging to a
YAC-contig overspanning the HMGI-C gene as described in the
Legenda of figure 2. Three of these cosmids (27E12, 185H2,
142H1) are mapping to the third intron of HMGI-C, whereas
5 cosmids 260C7 and 245E8 are localized at the 3' or the 5'
end respectively. The slides were analyzed using a Zeiss
(Zeiss, Oberkochem, Germany) Axioplan fluorescence
microscope. Results were processed and recorded with the
Power Gene Karyotyping System (PSI, Halladale, Great
10 Britain). Rapid amplification of cDNA ends (RACE) was
performed as described in one of the former examples.

3. Results

All four tumors showed the same type of
15 cytogenetic abnormality, i.e. the presence of 47 chromosomes
including two apparently normal chromosomes 12 and an
additional derivative 14 der(14)t(12;14)(q14-15;q24) but
without a corresponding der(12). Since the 3'-5' orientation
of the HMGI-C is towards the centromere a single break
20 within the HMGI-C gene would have led to the loss of its 5'
part along with the loss of the der(12). We have therefore
performed a series of FISH experiments in order to determine
the breakpoints more precisely. Using the five cosmids
260C7, 27E12, 185H2, 142H1, and 245E8 hybridization signals
25 of the same intensity were observed at both normal
chromosomes 12 and at the additional der(14). The FISH
results revealed that in all four cases chromosomal
breakpoints were located 5' of the HMGI-C gene.

The breakpoint assignment in all four cases 5' of
30 the HMGI-C gene fits well with the results of the RACE-PCR.
In addition to the normal HMGI-C transcripts we were able to
detect aberrant transcripts in all three tumors. Sequences
showed that they were not derived from chromosome 14 but
from intron 3 of HMGI-C probably due to cryptic splice
35 sites. However, the RACE results revealed that there was
indeed HMGI-C expression in all four cases.

47

EXAMPLE 11

Re-differentiation of leukemic cells

1. Introduction

Expression of the HMGI-C gene is frequently
5 strongly elevated in a wide variety of tumors, solid tumors
as well as leukemias. It was speculated that the HMGI-C
protein might play a key role in transformation of cells.
This example shows that expression of the HMGI-C gene can be
strongly reduced by expressing antisense HMGI-C sequences
10 and that reduction of HMGI-C levels in tumor cells results
in reversion of the transformed phenotype. Thus the
expression or administration of antisense molecules can be
successfully applied therapeutically.

15 2. Materials and methods

2.1. Tumor cell lines

Tumor cell lines were generated from a primary
malignant salivary gland tumor and a primary breast
carcinoma. Cell lines were established as described by
20 Kazmierczak, B., Thode, B., Bartnitzke, S., Bullerdiek, J.
and Schloot, W., "Pleomorphic adenoma cells vary in their
susceptibility to SV40 transformation depending on the
initial karytotype.", Genes Chrom. Cancer 5:35-39 (1992).

25 2.2. Assay of the transformed state

Soft agar colony assays were performed as
described by Macpherson and Montagnier, "Agar suspension
culture for the selective assays of cells transformed by
polyoma virus." Virology 23, 291-294 (1964).
30 Salivary gland and breast tumor cells were
propagated in TC199 culture medium with Earle's salts,
supplemented with 20% fetal bovine serum (GIBCO), 200 IU/ml
penicillin, and 200 μg/ml streptomycin.
Tumorigenicity of the transfected salivary gland
35 (AD64) and breast cell lines was tested by injecting cells
subcutaneously into athymic mice.

48

2.3. Transfection assay

        Transfections were performed using various
protocols, namely:

1. The calcium phosphate procedure of Graham and Van der Eb
5 ("A new technique for the assay of the infectivity of human
adenovirus." Virology 52, 456-467 (1973)).

2. Lipofection: Transfections were carried out using
liposome-mediated DNA transfer (lipofectamine, GibcoBRL)
according to the guidelines of the manufacturer.
10

2.4. Antisense constructs

        Sense and antisense constructs of the HMGI-C gene
were obtained by inserting human HMGI-C cDNA sequences in
both the sense and antisense orientation in expression
15 vectors under the transcriptional control of various
promoter contexts, e.g. the long terminal repeat of Moloney
murine leukemia virus, a CMV promoter, or the early promoter
of SV40. For example, the CMV/HMGI-C plasmid was constructed
by cloning a human HMGI-C cDNA fragment containing all
20 coding sequences of human HMGI-C in pRC/CMV (Invitogen)
allowing expression under control of the human
cytomegalovirus early promoter and enhancer, and selection
for G418 resistance.

25 3. Results

3.1. Reversion of the transformed phenotype

        Reversion of the transformed phenotype was
observed in breast and salivary gland tumors cells after
induction of antisense HMGI-C expression in these tumor
30 cells. A strong reduction in tumorigenicity was observed as
measured by soft agar colony assay and in vivo in athymic
mice. Immunoprecipitation and Western blot analysis
indicated a strong reduction of HMGI-C protein levels in the
cells expressing antisense HMGI-C sequences. Therefore, this
35 approach can be used therapeutically in tumors with
involvement of HMGI-C.

EXAMPLE 12

Animal tumor models involving HMGI-C as tools in _in vivo_
therapeutic drug testing.

On the basis of the acquired HMGI-C knowledge,
5 animal tumor models can be developed as tools for _in vivo_
drug testing. To achieve this objective (for instance for
uterine leiomyoma), two approaches can be used, namely gene
transfer (generation of transgenic animals) on the one hand
and gene targeting technology (mimicking _in vivo_ of a
10 specific genetic aberration via homologous recombination in
embryonic stem cells (ES cells)) on the other.

These technologies allow manipulation of the
genetic constitution of complex living systems in specific
and pre-designed ways. For extensive technical details, see
15 B. Hogan, R. Beddington, F. Constantini, and E. Lacy; In:
Manipulating the mouse embryo, A Laboratory Manual. Cold
Spring Harbor Press, 1994; ISBN 0-87969-384-3.

To aim at the inactivation or mutation of the
HMGI-C gene, specifically in selected cell types and
20 selected moments in time, the recently described _Cre/LoxP_
system can be used (Gu, H. et al. Deletion of a DNA
polymerase ß gene segment in T cells using cell type-
specific gene targeting. Science 265, 103-106, 1994). The
Cre enzyme is a recombinase from bacteriophage P1 whose
25 physiological role is to separate phage genomes that become
joined to one another during infection. To achieve so, Cre
lines up short sequences of phage DNA, called loxP sites and
removes the DNA between them, leaving one loxP site behind.
This system has now been shown to be effective in mammalian
30 cells in excising at high efficiency chromosomal DNA.
Tissue-specific inactivation or mutation of a gene using
this system can be obtained via tissue-specific expression
of the Cre enzyme.

As an example, the development of animal model
35 systems for uterine leiomyoma using a member of the MAG gene
family will be outlined below, such that the models will be
instrumental in _in vivo_ testing of therapeutic drugs.

Two approaches may be followed:

a) <u>in vivo</u> induction of specific genetic aberrations as
observed in human patients ((conditional) gene (isogenic)
targeting approach); and

b) introduction of DNA constructs representative for the
5 genetic aberrations observed in patients (gene transfer
approach).

        DNA constructs to be used in gene transfer may be
generated on the basis of observations made in patients
suffering from uterine leiomyoma as far as structure and
10 expression control are concerned; e.g. HMGI-C fusion genes
with various translocation partner genes, especially the
preferential translocation partner gene of chromosome 14
located in the YAC contig represented by CEPH YACs 6C3,
89C5, 308H7, 336H12, 460A6, 489F4, 902F10, 952F5, 958C2,
15 961E1, and 971F5, truncated genes encoding basically the
three DNA binding domains of HMGI-C, and complete HMGI-C or
derivatives of HMGI-C under control of a strong promoter.

EXAMPLE 13
20 The preparation of antibodies against HMGI-C
        One type of suitable molecules for use in
diagnosis and therapy are antibodies directed against the
MAG genes. For the preparation of rabbit polyclonal
antibodies against HMGI-C use was made of the following
25 three commercially available peptides:
(H-ARGEGAGQPSTSAQGQPAAPAPQKR)8-<u>M</u>ultiple <u>A</u>ntigen <u>P</u>eptide
(H-SPSKAAQKKAEATGEKR)8-MAP
(H-PRKWPQQVVQKKPAQEE)8-MAP
obtainable from Research Genetics Inc., Huntsville, AL, USA.
30 The polyclonal antibodies were made according to standard
techniques.

TABLES

TABLE I

ANALYSIS OF YAC CLONES

CEPH-Code	Size (kb)	Landmark left	#	Landmark right	#	Chimeric
183F3	715			[RM10]		YES (L + R)
70E1	450	RM29	U27125			ND
95F1	390			RM30	U29054	ND
201H7	320	RM13	U29051	RM14	U29053	ND
186G12	320					ND
354B6	280					YES (R)
126G9	410					ND
258F11	415	RM4	U29052			ND
320F6	290	RM5	U29050	RM21	U29047	ND
234G11	475	RM7	U29046			ND
375H5	290					ND
252E10	510	[RM15]		RM16	U29048	YES (L)
181C8	470			RM26	U29045	ND
107D1	345	RM31	U29043			ND
499C5	320	RM44	U29044	RM46	U29037	ND
340B6	255					ND
532C12	400	RM45	U29041			ND
138C5	510	[RM59]		RM65	U29042	YES (L)
145F2	490	RM60	U29030	RM66	U29040	ND
105E8	340	RM57	U29033	RM63	U29038	ND
55G1	365	RM56	U29031	RM62	U29039	ND
103G7	370	RM85	U29025	RM80	U29036	ND
295B10	295	RM77	U29035	RM81	U29026	ND
338C2	200	RM78	U29034	RM82	U29029	ND
391C12	160	[RM79]		RM83	U29027	YES (L)
476A11	225	[RM87]		RM84	U29032	YES (L)
138F3	460	RM90	U29028	RM91	U29019	ND
226E7	500	RM48	U29024	RM54	U29015	ND
499E9	375	RM51	U29016			YES (R)
312F10	580	[RM50]		RM69	U29021	YES (L)
825G7	950					ND
34B5	315	RM88	U29020	RM89	U29013	ND
94A7	610					YES (R)
305B2	660					YES (L)
379H1	280	RM104	U29014	RM105	U29009	ND
444E6	350	RM92	U29017	RM93	U29010	ND
446H3	370	RM94	U29011	RM95	U29018	ND
403B12	380					ND
261E5	500	RM102	U29012	RM103	U26689	ND
78B11	425					ND
921B9	1670					ND
939H2	1750					ND
188H7	360					ND
142F4	390					ND
404E12	350					ND
164A3	375					ND
244B12	415	RM106	U29007	RM107	U29008	ND
275H4	345	RM108	U29004	RM109	U29005	ND
320F9	370					ND
51F8	450					ND
242A2	160	CH1	U29006			

TABLE I (continued)

ANALYSIS OF YAC CLONES

253H1	400					ND
303F11	320					ND
322C8	410			CH2	U29003	ND
208G12	370	RM96	U29002	RM97	U27135	ND
341C1	270	RM98	U28647	RM99	U27130	ND
354F1	270					ND
452E1	270	CH5	U27136			ND
41A2	310					ND
934D2	1370					ND
944E8	1290			CH8	U25792	ND
2G11	350					ND
755D7	1390					YES (L)
365A12	370					ND
803C2	1080					ND
210C1	395	RM70	U28998	RM86	U27133	ND
433C8	360	RM73	U29000	RM76	U27132	ND
402A7	500	RM41	U28994	[RM42]		YES (R)
227E8	465	RM53	U27134	RM55	U28996	ND
329F9	275	RM72	U28793	RM75	U28997	ND
261E6	395	[RM71]		RM74	U28995	YES (L)
348F2	370			[RM136]		YES (R)
6F3	320	RM35	U27140	RM36	U27141	
59F12	430	RM34	U28794	RM33	U27131	
265H3	300			RM40	U28999	

YAC clones were isolated from CEPH YAC libraries as described in Materials and Methods. ND: not detected by methods used. Landmarks not mapping within the 6 Mb contig have been bracketed. GenBank accession numbers are given (#).

TABLE II

PCR Primers

STS name (STS 12-)	Nucleotide sequence 5'-3'	Product size (bp)	T_m (°C)
CH1	TGGGACTAACGGATTTTCAA TGTGGTTCATTCATGCATTA	213	58
CH2	TCCATCATCATCTCAAAACA CTCTACCAAATGGAATAAACAG	145	58
CH5	GCAGCTCAGGCTCCTTCCCA TGGCTTCCTGAAACGCGAGA	143	58
CH8	TCTCCACTGCTTCCATTCAC ACACAAAACCACTGGGGTCT	147	58
CH9	CAGCTTTGGAATCAGTGAGG CCTGGGGAAGAGGAGTAAAG	262	58
RM1	GAGCTTCCTATCTCATCC ATGCTTGTGTGTGAGTGG	308	60
RM4	TTTGCTAAGCTAGGTGCC AGCTTCAAGACCCATGAG	236	60
RM5	CAGTTCTGAGACTGCTTG TAATAGCAGGGACTCAGC	324	60
RM7	CTTGTCTCATTCTTTTAAAGGG CACCCCTTTTTAGATCCTAC	533	58
RM13	GAATGTTCATCACAGTGCTG AATGTGAGGTTCTGCTGAAG	±500	58
RM14	TTCTCATGGGGTAAGGACAG AAAGCTGCTTATATAGGGAATC	158	58
RM16	CCTTGGCTTAGATATGATACAC GCTCTTCAGAAATATCCTATGG	252	58
RM21	CCTTAGCAGTTGCTTGTCTG TCGTCACAGGACATAGTCAC	290	58
RM26	TCTATGGTATGTTATACAAGATG CAGTGAGATCCTGTCTCTA	102	58
RM31	TCTGTGATGTTTTAAGCCACTTAG AATTCTGTGTCCCTGCCACC	239	56
RM33	ATTCTTCCTCACCTCCCACC AATCTGCAGAGAGGTCCAGC	±600	60
RM34	AATTCTCCATCTGGGCCTGG GAACGCTAAGCATGTGGGAG	±600	60
RM36	CTCCAACCATGGTCCAAAAC GACCTCCAGTGGCTCTTAG	296	60
RM46	ACCATCAGATCTGGCACTGA TTACATTGGAGCTGTCATGC	241	57
RM48	TCCAGGACATCCTGAAAATG AGTATCCTGCACTTCTGCAG	391	58
RM51	GATGAACTCTGAGGTGCCTTC TCAAACCCAGCTTTGACTCC	311	60
RM53	GTCTTCAAAACGCTTTCCTG TGGTTTGCATAATGGTGATG	333	60

54

TABLE II (continued)

PCR Primers

STS name (STS 12-)	Nucleotide sequence 5'-3'	Product size (bp)	T_{am} (°C)
RM60	TACACTACTCTGCAGCACAC TCTGAGTCAATCACATGTCC	94	58
RM69	CTCCCCAGATGATCTCTTTC CGGTAGGAAATAAAGGAGAG	236	58
RM72	TATTTACTAGCTGGCCTTGG CATCTCAGGCACACACAATG	101	62
RM76	ATTCAGAGAAGTGGCCAAGT GGGATAGGTCTTCTGCAATC	496	58
RM85	TCCAACAATACTGAGTGACC TCCATTTCACTGTAGCACTG	435	58
RM86	GTAATCAACCATTCCCCTGA AAAATAGCTGGTATGGTGGC	203	56
RM90	ACTGCTCTAGTTTTCAAGGA AATTTACCTGACAGTTTCCT	257	58
RM93	GCATTTGACGTCCAATATTG ATTCCATTGGCTAACACAAG	347	60
RM98	GCAAAACTTTGACTGAAACG CACAGAGTATCGCACTGCAT	356	58
RM99	AAGAGATTTCCCATGTTGTG CTAGTGCCTTCACAAGAACC	240	58
RM103	AATTCTTGAGGGGTTCACTG TCCACACTGAGAGCTTTTCA	199	60
RM108	GTGGTTCTGTACAGCAGTGG TGAGAAAATGTCTGCCAAAT	439	60
RM110	GCTCTACCAGGCATACAGTG ATTCCTAGCATCTTTTCACG	328	58
RM111	ATATGCATTAGGCTCAACCC ATCCCACAGGTCAACATGAC	312	58
RM130	ATCCTTACATTTCCAGTGGCATTCA CCCAGAAGACCCACATTCCTCAT	336	58
RM131	TTTTAAGTTTCTCCAGGGAGGAGAC AATAGGCTCTTTGGAAAGCTGGAGT	226	58
RM132	TCTCAGCTTAATCCAAGAAGGACTTC GGCATATTCCTCAACAATTTATGCTT	376	58
RM133	TGGAGAAGCTATGGTGCTTCCTATG TGACAAATAGGTGAGGGAAAGTTGTTAT	225	58
EST01096	TCACACGCTGAATCAATCTT CAGCAGCTGATACAAGCTTT	186	58
IFNG	TGTTTTCTTTCCCGATAGGT CTGGGATGCTCTTCGACCTC	150	52
Rap1B	CCATCCAACATCTTAAATGGAC CAGCTGCAAACTCTAGGACTATT	149	58

STSs were isolated as described in Materials and Methods, or retrieved from literature for EST01096, IFNG, and Rap1B.

Table 3

Genome Data Base accession numbers (D-numbers) of the various sequences indicated in Figure 1.

Genome Data Base

pcr	locus_symbol	pcr	pcr_gdb_id	(41 rows effected) locus	locus_gdb_id
CH1-lower/CH1-upper	D12S1484	CH1-lower/CH1-upper	G00-595-292	D12S1484	G00-595-415
CH2-lower/CH2-upper	D12S1485	CH2-lower/CH2-upper	G00-595-295	D12S1485	G00-595-416
CH5-lower/CH5-upper	D12S1486	CH5-lower/CH5-upper	G00-595-298	D12S1486	G00-595-417
CH8-lower/CH8-upper	D12S1487	CH8-lower/CH8-upper	G00-595-301	D12S1487	G00-595-418
CH9-lower/CH9-upper	D12S1488	CH9-lower/CH9-upper	G00-595-304	D12S1488	G00-595-419
EH2-lower/EH2-upper	D12S1489	EH2-lower/EH2-upper	G00-595-307	D12S1489	G00-595-420
EH3-lower/EH3-upper	D12S1490	EH3-lower/EH3-upper	G00-595-310	D12S1490	G00-595-421
EH4-lower/EH4-upper	D12S1491	EH4-lower/EH4-upper	G00-595-313	D12S1491	G00-595-422
RH13-lower/RH13-upper	D12S1492	RH13-lower/RH13-upper	G00-595-316	D12S1492	G00-595-423
RH14-lower/RH14-upper	D12S1493	RH14-lower/RH14-upper	G00-595-319	D12S1493	G00-595-424
RH16-lower/RH16-upper	D12S1494	RH16-lower/RH16-upper	G00-595-322	D12S1494	G00-595-425
RH25-lower/RH25-upper	D12S1507	RH26-lower/RH26-upper	G00-595-325	D12S1495	G00-595-426
RH26-lower/RH26-upper	D12S1495	RH-29-lower/RH29-upper	G00-595-328	D12S1496	G00-595-427
RH31-lower/RH31-upper	D12S1497	RH31-lower/RH31-upper	G00-595-331	D12S1497	G00-595-428
RH33-lower/RH33-upper	D12S1498	RH33-lower/RH33-upper	G00-595-334	D12S1498	G00-595-429
RH34-lower/RH34-upper	D12S1499	RH34-lower/RH34-upper	G00-595-337	D12S1499	G00-595-430
RH36-lower/RH36-upper	D12S1500	RH36-lower/RH36-upper	G00-595-340	D12S1500	G00-595-431
RH46-lower/RH46-upper	D12S1501	RH46-lower/RH46-upper	G00-595-343	D12S1501	G00-595-432
RH48-lower/RH48-upper	D12S1502	RH48-lower/RH48-upper	G00-595-346	D12S1502	G00-595-433
RH51-lower/RH51-upper	D12S1503	RH51-lower/RH51-upper	G00-595-349	D12S1503	G00-595-434
RH53-lower/RH53-upper	D12S1504	RH53-lower/RH53-upper	G00-595-352	D12S1504	G00-595-435
RH60-lower/RH60-upper	D12S1505	RH60-lower/RH60-upper	G00-595-355	D12S1505	G00-595-436
RH69-lower/RH69-upper	D12S1506	RH69-lower/RH69-upper	G00-595-358	D12S1506	G00-595-437
RH72-lower/RH72-upper	D12S1508	RH25-lower/RH25-upper	G00-595-361	D12S1507	G00-595-438
RH76-lower/RH76-upper	D12S1509	RH72-lower/RH72-upper	G00-595-364	D12S1508	G00-595-439
RH85-lower/RH85-upper	D12S1510	RH76-lower/RH76-upper	G00-595-367	D12S1509	G00-595-440
RH86-lower/RH86-upper	D12S1511	RH85-lower/RH85-upper	G00-595-370	D12S1510	G00-595-441
RH90-lower/RH90-upper	D12S1512	RH86-lower/RH86-upper	G00-595-373	D12S1511	G00-595-442
RH93-lower/RH93-upper	D12S1513	RH90-lower/RH90-upper	G00-595-376	D12S1512	G00-595-443
RH98-lower/RH98-upper	D12S1514	RH93-lower/RH93-upper	G00-595-379	D12S1513	G00-595-444
RH99-lower/RH99-upper	D12S1515	RH98-lower/RH98-upper	G00-595-382	D12S1514	G00-595-445
RH-29-lower/RH29-upper	D12S1496	RH99-lower/RH99-upper	G00-595-385	D12S1515	G00-595-446
RH103-lower/RH103-upper	D12S1516	RH103-lower/RH103-upper	G00-595-388	D12S1516	G00-595-447
RH108-lower/RH108-upper	D12S1517	RH108-lower/RH108-upper	G00-595-391	D12S1517	G00-595-448
RH110-lower/RH110-upper	D12S1518	RH110-lower/RH110-upper	G00-595-394	D12S1518	G00-595-449
RH111-lower/RH111-upper	D12S1519	RH111-lower/RH111-upper	G00-595-397	D12S1519	G00-595-450
RH121-lower/RH121-upper	D12S1520	RH121-lower/RH121-upper	G00-595-400	D12S1520	G00-595-451
RH130-lower/RH130-upper	D12S1521	RH130-lower/RH130-upper	G00-595-403	D12S1521	G00-595-452
RH131-lower/RH131-upper	D12S1522	RH131-lower/RH131-upper	G00-595-406	D12S1522	G00-595-453
RH132-lower/RH132-upper	D12S1523	RH132-lower/RH132-upper	G00-595-409	D12S1523	G00-595-454
RH133-lower/RH133-upper	D12S1524	RH133-lower/RH133-upper	G00-595-412	D12S1524	G00-595-455

56

TABLE 4

FISH mapping of chromosome 12 breakpoints in primary benign solid tumors
to a subregion of MAR

Tumor type	Breakpoint within MAR	Fraction of tumors with breakpoints within main breakpoint cluster region[*]
Lipoma	6/6	6/6
Pleomorphic salivary gland adenoma	7/7	5/7
Uterine leiomyoma	7/8	7/8
Hamartoma of the breast	1/1	1/1
Fibroadenoma of the breast	1/1	1/1
Hamartoma of the lung	8/9	8/9
Angiomyxoma	1/1	1/1

[*] Tumor samples were collected and analyzed at the histopathology and cytogenetics facilities of the University of Bremen. A mixture of cosmid clones 27E12 and 142H1 was used as molecular probe in FISH analysis.

Table 5-1

Clone	Tumor/Cell Line	Diversion Point exon/intron 3 (DBD3)	Nuc. Sequences (10b) TAGGAAATGG	GTGAGTAATA	Chrom. RACE	source cytogen.	poly(A) signal	#A's	primer-set
pCH108	L1-14/SV40	after DBD3	TAGGAAATGG	AATACTCTGG	12	3q27	AGTAAA	26	?
pCH113	L1-538/SV40	after DBD3	TAGGAAATGG	AATACTCTGG	12	3q27	AGTAAA	26	?
pCH234	#2528-90	after DBD3	TAGGAAATGG	AATACTCTGG	12	?	(AGTAAA)	17	
pCH259	#2344-94	after DBD3	TAGGAAATGG	AATACTCTGG	12	?	?	?	
pCH260	#2344-94	after DBD3	TAGGAAATGG	AATACTCTGG	12	?	?	?	
pCH148	#192	after DBD3	TAGGAAATGG	CCTACTATTG	12	N.T.	?	?	
pCH245	#560 92	after DBD3	TAGGAAATGG	CCTACTATTG	12	12.	AATAAA	18	---
pCH247	#560 92	after DBD3	TAGGAAATGG	CCTACTATTG	12		AATAAA	17	
pCH261	#2344-94	after DBD3	TAGGAAATGG	CCTACTATTG	12		AATAAA	53	
pCH212	#1321-89 ?	after DBD3	TAGGAAATGG	GGAAGTGTGA	12		AATAAA	27	
pCH213	#1321-89 ?	after DBD3	TAGGAAATGG	GGAAGTGTGA	12		AATAAA	16	
pCH228	#1321-89	after DBD3	TAGGAAATGG	GGAAGTGTGA	12		?	?	
pCH216	#2100-89	after DBD3	TAGGAAATGG	AACACAGGAC	12		N.D.	13	
pCH229	#2100-89	after DBD3	TAGGAAATGG	AACACAGGAC	12		AATAAA	12	
pCH232	#2100-89	after DBD3	TAGGAAATGG	AACACAGGAC	12		AATAAA	15	
pCH177	#367	after DBD3	TAGGAAATGG	GTTTAATATT	12	2p21	AATAAA	25	
pCH191	#25 ?	after DBD3	TAGGAAATGG	GTTTAATATT	12	?	AATAAA	29	177
pCH210	#837-88	after DBD3	TAGGAAATGG	AAGAGGCAG	12	3	AATAAA	49	(177)
pCH211	#837-88	after DBD3	TAGGAAATGG	AAGAGGCAG	12	3	AATAAA	22	
pCH230	#2100-89	after DBD3	TAGGAAATGG	TAGGAGGTAG	12		AATAAA	27	
pCH233	#2100-89	after DBD3	TAGGAAATGG	TAGGAGGTAG	12		AATAAA	18	
pCH111	L1-501/SV40	after DBD3	TAGGAAATGG	GGTGGCCATT	12	3	AATAAA	33	111-AB
pCH112	L1-501/SV40	after DBD3	TAGGAAATGG	GGTGGCCATT	12	3q27	AATAAA	33	111-AB
pCH114	L1-538/SV40	after DBD3	TAGGAAATGG	GACAATCTAC	12	3q27	(CATAAA)	24	115-AB
pCH115	L1-538/SV40	after DBD3	TAGGAAATGG	GACAATCTAC	12	3q27	(CATAAA)	24	115-AB
pCH147	#192	after DBD3	TAGGAAATGG	GTACAGAAGA	12	12	AATAAA	23	147
pCH153	#203	after DBD3	TAGGAAATGG	GGGCATTCAG	12	9	AATAAA	22	
pCH169	#294	after DBD3	TAGGAAATGG	GCAGTCTGTA	12	N.T.	AATAAA	26	169
pCH172	#294	after DBD3	TAGGAAATGG	TCTGTATCCT	12	8q22	AATAAA	24	172-AB
pCH173	#294	after DBD3	TAGGAAATGG	ACACACTTCC	12	8q22-qter	AATAAA	12	173-AB
pCH174	#294	after DBD3	TAGGAAATGG	ATATTATTGA	12	? 8q22	AATAAA	15	174-AB
pCH110	LM-30.1/SV40	after DBD3	TAGGAAATGG	GAGGAGTTTT	12	14	AATAAA	17	110-AB
pCH164	Myo168.1	after DBD3	TAGGAAATGG	TAACACAGGA	12	x	(GATAAA)	30	164-AB
pCH165	Myo168.1	after DBD3	TAGGAAATGG	TTACCTGCTC	12	x	(AATAAC)	30	165-AB
pCH168	Myo19G.4	after DBD3	TAGGAAATGG	GCTGGAGTGC	12	14	(CATAAA)	23	168
pCH209	#837-88 ?	after DBD3	TAGGAAATGG	GTCTCCTCCC	12		AATAAA	19	
pCH217	#2100-89 ?	after DBD3	TAGGAAATGG	TTTNTCTCTT	12		ATTAAA	22	
pCH219	#2100-89 ?	after DBD3	TAGGAAATGG	AGTCCAAGAA	12		AATAAA	17	
pCH220	#3391-90	after DBD3	TAGGAAATGG	CCAAACTCTG	12		(CATAAA)	28	
pCH223	#CG592	after DBD3	TAGGAAATGG	CTCCAGAAAC	12		AATAAA	24	
pCH226	#3100-88	after DBD3	TAGGAAATGG	AACTTCTTGA	12		?	?	
pCH231	#2100-89	after DBD3	TAGGAAATGG	GAATGTCAGA	12		AATAAA	24	
pCH246	#568-92	after DBD3	TAGGAAATGG	CCTGGAAGCT	12		AATAAA	?	
pCH248	#568-92	after DBD3	TAGGAAATGG	ATGGAGTCTC	12		AATAAA	40	
pCH249	#2617-93	after DBD3	TAGGAAATGG	ATGGAGTCTC	12		AATAAA	19	
pCH251	#2617-93	after DBD3	TAGGAAATGG	ATGGAGTCTC	12		N.D.	19	
pCH250	#2617-93	after DBD3	TAGGAAATGG	TTCCAGATAC	12		N.D.	13	
pCH252	#2617-93	after DBD3	TAGGAAATGG	TTCCAGATAC	12		N.D.	13	

Table 5-2

Clone	Tumor/Cell line	Diversion Point	Nuc. Sequences (10b)		Chrom. sources		poly(A) signal	#A's	primer-set
			exon/intron 4 GCCTGCTCAG	(SPACER) GTAAGACATA	RACE	cytogen.			
pCII109	LN30.1/SV40	after spacer	GCCTGCTCAG	GTCAATGTTG	12	12	AATAAA	17	109-AB
pCII238	#2778-93	after spacer	GCCTGCTCAG	GTCAATGTTG	12	14	AATAAA	17	109-AB
pCII244	#2162-91	after spacer	GCCTGCTCAG	GTCAATGTTG	12	14	AATAAA	17	109-AB
pCII254	#2776-93	after spacer	GCCTGCTCAG	GTCAATGTTG	12	14	AATAAA	17	109-AB
pCH203	#2528-90	after spacer	GCCTGCTCAG	GTCAATGTTG	12	14	N.D.	19	109-AB
pCII199	#CG575	after spacer	GCCTGCTCAG	TCCTGGTACC	12	(NF1")	(AATAAA)	15	
pCII222	#CG575	after spacer	GCCTGCTCAG	TCCTGGTACC	12	(NF1")	N.D.	16	
pCII206	#CG575	after spacer	GCCTGCTCAG	TCCTGGTACC	12	(NF1")	(AATAAA)	16	
pCII175	#275	after spacer	GCCTGCTCAG	TCTTTCAGAT	12 ?	1	AATAAA	15	175-AB
pCII237	#2617-93	after spacer	GCCTGCTCAG	TCTTTCAGAT	12 ?		AATAAA	16	
pCII207	#2540-87	after spacer	GCCTGCTCAG	AATTACCTCT	12	12	AATAAA	18	
pCH239	#2344-94	after spacer	GCCTGCTCAG	AATTACCTCT	12		AATAAA	17	
pCII116	L1-14/SV40	after spacer	GCCTGCTCAG	GACTGACTCA	12	3	(AATAAA)	19	116-AB
pCII184	Myo163.1	after spacer	GCCTGCTCAG	TATTCCTGAA	12	14	AATAAA	47	184-AU
pCII208	#2540-87	after spacer	GCCTGCTCAG	GTCAATGTTG	12	12	AATAAA	15	
pCH224	#2540-87	after spacer	GCCTGCTCAG	AATTACCTCT	12	12	AATAAA	28	
pCII201	#837-88	after spacer	GCCTGCTCAG	AATTACCTCT	12		AATAAA	7	
pCII236	#2162-91	after spacer	GCCTGCTCAG	GCTTTTTCAA	12		AATAAA	14	
pCII243	#2162-91	after spacer	GCCTGCTCAG	GTAAGAAAC	12		?		
pCII227	#183-89	after spacer	GCCTGCTCAG	GACTGACTAC	12	12	(AATAGA)		

3'-untranslated region (various positions)

Clone	Tumor/Cell line	Diversion Point	Nuc. Sequences (10b)		Chrom. sources		poly(A) signal	#A's	primer-set
pCII193	#58	within 3'-UTR	TATCCTTTCA	AAGTCAAGAG	12	8q22-qter8q24	AATAAA	23	(195-AB)
pCH194	#58	within 3'-UTR	TATCCTTTCA	AAGTCAAGAG	12	8q22-qter8q24	AATAAA	34	(195-AB)
pCII195	#192	within 3'-UTR	TATCCTTTCA	AAGTCAAGAG	12	8	AATAAA	23	195-AB
pCII196	#192	within 3'-UTR	TATCCTTTCA	AAGTCAAGAG	12	12	AATAAA	>17	195-AB
pCH189	Myo192.1	within 3'-UTR	TCTTTCCACT del CCCTGT ATACCACTTA	TTTTAAACA	12 ?				
pCII117	Ad-312/SV40	within 3'-UTR	TTGCCATGGT	AATCTGAAAT	12	N.T.[1] 2/3	AATAAA	23	117-AB
pCII253	#2617-93	within 3'-UTR	CACTTCATC	ATATGGCAAG	12	1p22 1p22	?	7	
pCII264	#568-92	within 3'-UTR	ATAAGGACTA	TCAGGCATCA	12		N.D.	19	
pCII270	#2528-90	within 3'-UTR	NCTTGTNAGC	TAGAGATTAG	12		(ACTAAA) N.D.	4	

N.T.: NOT TESTABLE:

N.T.[1]: LENGTH OF ECTOPIC SEQUENCE DOES NOT ALLOW DEVELOPMENT OF PRIMER-SET
N.T.[2]: ECTOPIC SEQUENCE IS MAINLY COMPOSED OF REPETITIVE SEQUENCES

N.D.: NOT DETECTED

LEGENDS TO THE FIGURES

Figure 1

Long range physical map of a 6 Mb region on the
5 long arm of human chromosome 12 deduced from a YAC contig
consisting of 75 overlapping CEPH YAC clones and spanning
the chromosome 12q breakpoints as present in a variety of
benign solid tumors. The long range physical map of the
composite genomic DNA covered by the YAC inserts is
10 represented by a black solid line with the relative
positions of the various restriction sites of rare cutting
enzymes indicated. DNA regions in which additional cutting
sites of a particular restriction enzyme might be found are
indicated by arrows. Polymorphic restriction endonuclease
15 sites are marked with asterisks. DNA markers isolated and
defined by others are depicted in green. DNA markers
obtained by us are shown in boxes and are labelled by an
acronym (see also Table I and II). The relative positions of
these DNA markers in the long range physical map are
20 indicated and those corresponding to particular YAC ends are
linked to these by a dotted line. Some of the DNA markers
have been assigned to a DNA interval and this is indicated
by arrows. For DNA markers in white boxes STSs have been
developed and primer sets are given in Table II. For those
25 in yellow boxes, no primer sets were developed. The DNA
intervals containing RAP1B, EST01096, or IFNG are indicated.
Where applicable, D number assignments are indicated. Below
the long range physical map, the sizes and relative
positions of the overlapping YAC clones fitting within the
30 consensus long range restriction map are given as solid blue
lines. DNA regions of YAC inserts not fitting within the
consensus long range restriction map are represented by
dotted blue lines. CEPH microtiter plate addresses of the
YAC clones are listed. The orientation of the YAC contig on
35 chromosome 12 is given. The relative positions of ULCR12 and
MAR are indicated by red solid lines labelled by the
corresponding acronyms. Accession numbers of STSs not listed
in Table I: CH9 (#U27142); RM1 (#U29049); RM110 (#U29022);

RM111 (#U29023); RM130 (#U27139); RM131 (#U29001); RM132
(#U27138); RM133 (#U27137). Restriction sites: B: BssHII; K:
KspI (=SacII); M: MluI; N: NotI; P: PvuI; Sf: SfiI.

5 **Figure 2**

Contig of overlapping cosmids, long range
restriction and STS map spanning a segment of MAR of about
445 kb. Contig elements are numbered and defined in the list
below. LL12NC01-derived cosmid clones are named after their
10 microtiter plate addresses. GenBank accession numbers (#) of
the various STSs are listed below. STSs are given in
abbreviated form; e.g. RM33 instead of STS 12-RM33. A 40 kb
gap between STSs "K" and "O" in the cosmid contig was
covered by λ clones (clones 38 and 40) and PCR products
15 (clones 37 and 39). The orientation of the contig on the
long arm of chromosome 12 is given as well as the order of
37 STSs (indicated in boxes or labelled with encircled
capital letters). The slanted lines and arrows around some
of the STS symbols at the top of the figure mark the region
20 to which the particular STS has been assigned. It should be
noted that the cosmid contig is not scaled; black squares
indicate STSs of cosmid ends whereas the presence of STSs
corresponding to internal cosmid sequences are represented
by dots. Long range restriction map: Bs: BssHII; K: KspI
25 (=SacII); M: MluI; N: NotI; P: PvuI; Sf: SfiI. At the bottom
of the figure, detailed restriction maps are shown of those
regions containing exons (boxes below) of the HMGI-C gene.
Noncoding sequences are represented by open boxes and coding
sequences by black boxes. Estimated sizes (kb) of introns
30 are as indicated. The relative positions of the translation
initiation (ATG) and stop (TAG) codons in the HMGI-C gene as
well as the putative poly-adenylation signal are indicated
by arrows. Detailed restriction map: B: BamHI; E: EcoRI; H:
HindIII. MAR: Multiple Aberration Region; DBD: DNA Binding
35 Domain.

1=140A3	11=142G8	21=124D8	31=59A1	41=128A2	51=65E6
2=202A1	12=154A10	22=128A7	32=101D8	42=142H1	52=196E1
3=78F11	13=163D1	23=129F9	33=175C7	43=204A10	53=215A8

```
   4=80C9     14=42H7    24=181C1   34=185H2   44=145E1   54=147G8
   5=109B12   15=113A5   25=238E1   35=189C2   45=245E8   55=211A9
   6=148C12   16=191H5   26=69B1    36=154B12  46=154F9   56=22D8
   7=14H6     17=248E4   27=260C7   37=pRM150  47=62D8    57=116B7
 5 8=51F8     18=33H7    28=156A4   38=pRM144  48=104A4   58=144D12
   9=57C3     19=50D7    29=27E12   39=PKXL    49=184A9
   10=86A10   20=68B12   30=46G3    40=pRM147  50=56C2
```

```
    A = STS 12-EM12  (#U27145)      I = STS 12-CH12  (#U27153)
 10 Q = STS 12-RM120 (#U27161)      B = STS 12-EM30  (#U27146)
    J = STS 12-EM10  (#U27154)      R = STS 12-RM118 (#U27162)
    C = STS 12-EM14  (#U27147)      K = STS 12-EM37  (#U27155)
    S = STS 12-RM119 (#U27163)      D = STS 12-EM31  (#U27148)
    L = STS 12-RM146 (#U27156)      T = STS 12-EM2   (#U27164)
 15 E = STS 12-CH11  (#U27149)      M = STS 12-RM145 (#U27157)
    U = STS 12-EM4   (#U27165)      F = STS 12-EM18  (#U27150)
    N = STS 12-RM151 (#U27158)      V = STS 12-EM3   (#U27166)
    G = STS 12-EM11  (#U27151)      O = STS 12-EM16  (#U27159)
    W = STS 12-EM15  (#U27167)      H = STS 12-CH10  (#U27152)
 20 P = STS 12-EM1   (#U27160)      X = STS 12-EM17  (#U27168)
```

```
    STS 12-CH5  (#U27136)      STS 12-CH9  (#U27142)
    STS 12-RM33 (#U27131)      STS 12-RM53 (#U27134)
    STS 12-RM76 (#U27132)      STS 12-RM86 (#U27133)
 25 STS 12-RM98 (#U26647)      STS 12-RM99 (#U27130)
    STS 12-RM103 (#U26689)     STS 12-RM130 (#U27139)
    STS 12-RM132 (#U27138)     STS 12-RM133 (#U27137)
    STS 12-RM151 (#U27158)
```

30

Figure 3

Schematic representation of FISH mapping data
obtained for tumor cell lines with chromosome 12q13-q15
aberrations, including 8 lipoma, 10 uterine leiomyoma, and 8
35 pleomorphic salivary gland adenoma cell lines in consecutive
experiments following our earlier FISH studies. Probes used
included phage clones pRM144 (corresponding STSs: RM86 and
RM130) and pRM147 (RM151), and cosmid clones 7D3 or 152F2
(RM103), 154F9 (CH9), 27E12 (EM11), 211A9 (RM33), 245E8
40 (RM53), 185H2 (RM76), 202A1 (RM98), 142H1 (RM99), 154B12
(RM132), and 124D8 (RM133). The DNA interval between RM33
and RM98 is estimated to be about 445 kb. Dots indicate
conclusive FISH experiments that were performed on metaphase
chromosomes of a particular cell line using as molecular

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probe, a clone containing the STS given in the box above.

Solid lines indicate DNA intervals to which a breakpoint of

a particular cell line was concluded to be mapping. Open

triangles indicate deletions observed during FISH analysis.

5 Open circles indicate results of FISH experiments on

metaphase chromosomes of Li-501/SV40 cells with

hybridization signals on a cytogenetically normal chromosome

3. The positions of chromosome 12 breakpoints of tumor cell

lines mapping outside MAR are indicated by arrows. The

10 molecularly cloned breakpoints of LM-30.1/SV40 and LM-

608/SV40 are indicated by asterisks. Breakpoints in various

uterine leiomyoma cell lines splitting cosmid 27E12 (EM11)

are indicated by "across".

15 **Figure 4**

3'-RACE product comprising the junction between

part of the HMGI-C gene and part of the LPP gene. The

primers used and the junction are indicated. The cDNA

synthesis was internally primed and not on the true poly(A)

20 tail.

Figure 5

Partial cDNA sequence of the LPP gene.

25 **Figure 6**

Amino acid sequence of the LPP gene. LIM domains

are boxed. The breaking point is indicated with an arrow.

Figure 7

Nucleotide sequence if HMGI-C (U28749). The
transcription start site indicated as proposed by
Manfioletti et al. [67] was arbitrarily chosen as a start
5 site. The sequence contains the complete coding sequence.

Figure 8

Gel of PCR products obtained as described in
Example 5.

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ANNEX 1

5 **Molecular Characterization of MAR, a Multiple Aberration Region on Human Chromosome Segment 12q13-q15 Implicated in Various Solid Tumors**

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20
Chromosome arm 12q breakpoints in seven cell lines
derived from primary pleomorphic salivary gland adenomas
were mapped by FISH analysis relative to nine DNA probes.
These probes all reside in a 2.8 Mb genomic DNA region of
25 chromosome segment 12q13-q15 and correspond to previously
published sequence-tagged sites (STS). Their relative
positions were established on the basis of YAC cloning
and long range physical and STS content mapping. The 12q
breakpoints of five of the cell lines were found to be
30 mapping within three different subregions of the 445 kb
DNA interval that was recently defined as the uterine
leiomyoma cluster region of chromosome 12 breakpoints
(ULCR12) between STS RM33 and RM98. All seven breakpoints
appeared to map within the 1.7 Mb DNA region between STS

35 _____

RM36 and RM103. Furthermore, the chromosome 12
breakpoints of three primary pleomorphic salivary gland
adenomas were also found to be mapping between RM36 and
RM103. Finally, FISH analysis of two lipoma cell lines
5 with 12q13-q15 aberrations pinpointed the breakpoints of
these to relatively small and adjacent DNA segments
which, as well as those of two primary lipomas, appeared
to be located also between RM36 and RM103. We conclude
from the observed clustering of the 12q breakpoints of
10 the three distinct solid tumor types that the 1.7 Mb DNA
region of the long arm of chromosome 12 between RM36 and
RM103 is a multiple aberration region which we designate
MAR. Genes Chromosom Cancer 12:296-303 (1995). © 1995
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INTRODUCTION

Chromosome translocations involving region q13-
q15 of chromosome 12 have been observed in a wide variety
20 of solid tumors (Mitelman, 1991). In subgroups of
cytogenetically abnormal uterine leiomyomas (Nilbert and
Heim, 1990; Pandis et al., 1991), pleomorphic salivary
gland adenomas (Sandros et al., 1990; Bullerdiek et al.,
1993), and benign adipose tissue tumors (Sreekantaiah et
25 al., 1991), 12q13-q15 aberrations are frequently
observed. In a recent study (Schoenmakers et al., 1994b),
we identified and molecularly characterized ULCR12, the
uterine leiomyoma cluster region of chromosome 12
breakpoints. In the present study, we focus on the
30 chromosome arm 12q breakpoints in pleomorphic adenoma of
the salivary glands, a benign epithelial tumor
originating from the major or minor salivary glands. It
is the most common type of salivary gland tumor and
accounts for almost 50% of all neoplasms in these organs.
35 About 85% of the tumors are found in the parotid gland,
10% in the minor salivary glands, and 5% in the
submandibular gland (Seifert et al., 1986). Although many
of these adenomas appear to have a normal karyotype,

cytogenetic studies have also revealed recurrent specific
chromosome anomalies (Sandros et al., 1990; Bullerdiek et
al., 1993). Besides chromosome 8 aberrations, often
translocations with a breakpoint in 8q12 with, as the
5 most common aberration, a t(3;8)(p21;q12), aberrations of
chromosome 12, usually translocations involving 12q13-
q15, are also frequent. Non-recurrent clonal
abnormalities have also been described. The frequent
involvement of region 12q13-q15 in distinct solid tumor
10 types suggests that this chromosomal region harbors
gene(s) that might be implicated in the evolution of
these tumors. Molecular cloning of the chromosome 12
breakpoints of these tumors and characterization of the
junction fragments may therefore lead to the
15 identification of such gene(s).

         On the basis of fluorescence in situ
hybridization (FISH) data, we have previously reported
that the chromosome 12 breakpoints in a number of cell
lines derived from primary pleomorphic salivary gland
20 adenomas (Kazmierczak et al., 1990; Schoenmakers et al.,
1994a), are located on the long arm of chromosome 12 in
the interval between loci D12S19 and D12S8 (Schoenmakers
et al., 1994a). This DNA interval has been estimated to
be about 7cM (Keats et al., 1989; Craig et al., 1993).
25 The interval containing the chromosome 12 breakpoints of
these tumor cells was narrowed further by showing that
all breakpoints mapped distally to the CHOP gene, which
is directly affected by the characteristic t(12;16)
translocation in myxoid liposarcomas (Aman et al., 1992;
30 Crozat et al., 1993; Rabbitts et al., 1993) and is
located between D12S19 and D12S8. In more recent studies
(Kools et al., 1995), the chromosome 12 breakpoint of
pleomorphic salivary gland adenoma cell line Ad-312/SV40
was pinpointed to a DNA region between sequence-tagged
35 sites (STSs) RM110 and RM111, which is less than 165 kb
in size. FISH evaluation of the chromosome 12 breakpoints
of the other pleomorphic salivary gland adenoma cell
lines indicated that they must be located proximally to

the one in Ad-312/SV40, at a distance of more than 800 kb
(Kools et al., 1995). These results pointed towards a
possible dispersion of the chromosome 12 breakpoints over
a relatively large genomic region on the long arm of
5 chromosome 12.

Here, we report physical mapping of the
chromosome 12 breakpoints in pleomorphic salivary gland
adenoma cells from primary tumors as well as established
tumor cell lines. The karyotypic anomalies observed in
10 the cells were all different but always involved region
q13-q15 of chromosome 12. Using DNA probes between D12S8
and CHOP, which corresponded to sequence-tagged sites
(STSs) of a long-range physical map of a 6 Mb DNA region
and were obtained during chromosome walking experiments,
15 we performed FISH experiments and defined more precisely
a major chromosome 12 breakpoint cluster region of
pleomorphic salivary gland adenoma. This breakpoint
cluster region appeared to overlap with ULCR12.
Furthermore, we tested whether 12q13-q15 breakpoints of
20 lipomas might also map within the same region as those of
pleomorphic salivary gland adenoma and uterine leiomyoma.

MATERIALS AND METHODS

25 **Primary Solid Tumors and Derivative Cell Lines.**
Primary solid tumors including pleomorphic
salivary gland adenomas, lipomas, and uterine leiomyomas
were obtained from the University Clinics in Leuven,
Belgium (Dr. I. De Wever); in Bremen, Germany (Dr. R.
30 Chille); in Krefeld, Germany (Dr. J. Haubrich); and from
the Institute of Pathology in Göteborg, Sweden (Dr. G.
Stenman). For cell culturing and subsequent FISH
analysis, tumor samples were finely minced, treated for
4-6 hours with 0.8% collagenase (Boehringer, Mannheim,
35 FRG), and processed further for FISH analysis according
to routine procedures.
Human tumor cell lines used in this study
included the previously described pleomorphic salivary

gland adenoma cell lines Ad-211/SV40, Ad-248/SV40, Ad-
263/SV40, Ad-295/SV40, Ad-302/SV40, AD-366/SV40, and Ad-
386/SV40 (Kazmierczak et al., 1990; Schoenmakers et al.,
1994a) and the lipoma cell lines Li-14/SV40 (Schoenmakers
5 et al., 1994a) and recently developed Li-538/SV40.
Chromosome 12 aberrations found in these cell lines are
listed in Table 1. Cells were propagated in TC199 culture
medium with Earle's salts supplemented with 20% fetal
bovine serum.

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TABLE 1. Chromosome 12 Abberrations in Primary Human
Solid Tumors and Cell Lines*

                                   Aberration
5   --
    Cell lines
    Ad-211/SV40   t(8;12)(q21;q13-q15)
    Ad-248/SV40   ins(12;6)(q15;q16q21)
    Ad-263/SV40   inv(12)(q15q24.1)
10 Ad-295/SV40   t(8;12;18)(p12;q14;p11.2)
    Ad-302/SV40   t(7;12)(q31;q14)
    Ad-366/SV40   inv(12)(p13q15)
    Ad-386/SV40   t(12;14)(q13-q15;q13-q15)

15 Li-14/SV40   t(3;12)(q28;q13)
    Li-538/SV40   t(3;12)(q27;q14)

    LM-5.1/SV40   t(12;15)(q15;q24)
    LM-30.1/SV40   t(12;14)(q15;q24)
20 LM-65/SV40   t(12;14)(q15;q24)
    LM-67/SV40   t(12;14)(q13-q15;q24)
    LM-100/SV40   t(12;14)(q15;q24)
    LM-605/SV40   ins(12;11)(q14;q21qter)
    LM-608/SV40   t(12;14)(q15;q24)
25 LM-609/SV40   t(12;14)(q15;q24)

    Primary tumors
    Ad-386   t(12;14)(q15;q11.2)
    Ad-396   t(3;12)
30 Ad-400   t(12;16)

    Li-166   t(12;12)
    Li-167   t(3;12)(q28;q14-q15)

35 LM-163.1   t(12;14)(q14;q24)
    LM-163.2   t(12;14)(q14-q24)
    LM-168.3   t(X;12)(q22;q15)
    LM-192   t(2;3;12)(q35;p21;q14)
    LM-196.4   t(12;14)(q14;q24)
40 --

*Ad, pleomorphic salivary gland adenoma; Li, lipoma;
uterine leiomyoma.

DNA probes.

In the context of a human genome project
focusing on the long arm of chromosome 12, we isolated
cosmid clones cRM33, cRM36, cRM51, cRM69, cRM72, cRM76,
5 cRM98, cRM103, and cRM133, from chromosome 12-specific
arrayed cosmid library LLNL12NC01 (Montgomery et al.,
1993). Further details of these cosmid clones have been
reported at the Second International Chromosome 12
Workshop (1994) and will be described elsewhere
10 (Kucherlapati et al., 1994). Briefly, initial screenings
were performed using a PCR-based screening strategy
(Green and Olson, 1990), followed by filter hybridization
analysis as the final screening step, as previously
described (Schoenmakers et al., 1994b). The cosmid clones
15 were isolated using STSs derived from YAC clones. STSs
were obtained upon rescue of YAC insert-ends using a
methodology involving vectorette-PCR followed by direct
solid phase fluorescent sequencing of the PCR products
(Geurts et al., 1994) or from inter-Alu PCR (Nelson et
20 al., 1989). Cosmid clones were grown and handled
according to standard procedures (Sambrook et al., 1989).

Cosmid clone cPK12qter, which maps to the
telomeric region of the long arm of chromosome 12 (Kools
25 et al., 1995) was used as a reference marker.

**Chromosome Preparations and Fluorescence In Situ
Hybridization.**

Metaphase spreads of the pleomorphic salivary
30 gland adenoma cell lines or normal human lymphocytes were
prepared as described before (Schoenmakers et al., 1993).
To unambiguously establish the identity of chromosomes
in the FISH experiments, FISH analysis was performed
after GTG-banding of the same metaphase spreads. GTG-
35 banding was performed essentially as described by Smit et
al. (1990). In situ hybridizations were carried out
according to a protocol described by Kievits et al.
(1990) with some minor modifications (Kools et al., 1994;

Schoenmakers et al., 1994b). Cosmid and YAC DNA was
labelled with biotin-11-dUTP (Boehringer Mannheim) or
biotin-14-dATP (BRL, Gaithersburg) as described before
(Schoenmakers et al., 1994b). Specimens were analyzed on
5   a Zeiss Axiophot fluorescence microscope using a FITC
filter (Zeiss). Results were recorded on Scotch (3M) 640
asa film.

RESULTS

10

**FISH Mapping of 12q Breakpoints in Cell Lines of
Pleomorphic Salivary Gland Adenoma.**
            In previous studies (Schoenmakers et al.,
1994a), we mapped the chromosome 12 breakpoints in a
15 number of pleomorphic adenomas of the salivary glands
relative to various DNA markers and established that
these were all located proximally to locus D12S8 and
distal to the CHOP gene. This region is somewhat smaller
than the 7 cM region encompassed by linkage loci D12S8
20 and D12S19 (Keats et al., 1989). Using YAC cloning, a
long range physical/STS map has been constructed covering
most of that 7 cM region, as recently reported
(Kucherlapati et al., 1994). Furthermore, numerous
genomic clones (cosmid clones) have been isolated and
25 their relative positions within this map established
(Kucherlapati et al., 1994). Nine of these cosmids,
including cRM33, cRM36, cRM51, cRM69, cRM72, cRM76,
cRM98, cRM103, and cRM133, were used in FISH studies to
establish the positions of the chromosome 12 breakpoints
30 of the seven cell lines derived from pleomorphic adenomas
of the salivary glands (Table 1). The relative mapping
order of these nine cosmid clones, which cover a genomic
region on the long arm of chromosome 12 of about 2.8 Mb,
is indicated in Figure 1 and the results of FISH studies
35 with the various cosmid probes are schematically
summarized in the same figure. As an illustration, FISH
results obtained with metaphase cells of cell line Ad-
295/SV40 using cRM76 and cRM103 as probes are shown in

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Figure 2. It should be noted that for the identification
of chromosomes, pre-FISH GTG-banding was used routinely.
On the basis of such banding, hybridization signals could
be assigned conclusively to chromosomes of known

5 identity; this was of major importance for cases with
cross- or background hybridization signals, as these were
occasionally observed. When GTG-banding in combination
with FISH analysis provided inconclusive results, either
because of weak hybridization signals or rather vague

10 banding, FISH experiments were performed with cosmid
clone cPK12qter (Kools et al., 1995) as a reference
probe.

    FISH analysis of metaphase chromosomes of each
of the seven pleomorphic salivary gland adenoma cell

15 lines with cosmid cRM103 revealed that this cosmid mapped
distal to the chromosome 12 breakpoints of all seven cell
lines studied here. Metaphase chromosomes of six of the
seven cell lines were also tested with probe cRM69 and,
in two cases, with cRM51. The results of the latter

20 experiments were always consistent with those obtained
with cRM103. Similar FISH analysis with cRM36 as probe
indicated that this probe mapped proximal to all the
breakpoints. These results were always consistent with
those obtained for five of the seven cell lines in

25 experiments using cRM72. Altogether, the results of our
FISH studies indicated that the chromosome 12 breakpoints
of all seven cell lines map between cRM36 and cRM103,
which spans a genomic region of about 1.7 Mb.

Fine Mapping of 12q Breakpoints in Cell Lines Derived from Pleomorphic Adenomas of the Salivary Glands.

For subsequent fine mapping of the chromosome
12 breakpoints of the seven pleomorphic salivary gland
5 adenoma cell lines, additional FISH studies were
performed, as schematically summarized in Figure 1. The
breakpoints of cell lines Ad-211/SV40, Ad-295/SV40, and
Ad-366/SV40 appeared to be located in the DNA region
between cRM76 and cRM133, which was estimated to be about
10 75 kb. The breakpoints of the four other cell lines were
found in different areas of the 1.7 Mb region between
cRM36 and cRM103. That of cell line Ad-248/SV40 in a DNA
segment of about 270 kb between cRM33 and cRM76, that of
Ad-263/SV40 in a DNA segment of about 1 Mb between cRM98
15 and cRM103, that of Ad-302/SV40 in a DNA segment of about
240 kb between cRM33 and cRM36, and that of Ad-386/SV40
in a DNA segement of about 100 kb between cRM98 and
cRM133. In conclusion, these results indicated that the
chromosome 12 breakpoints of most (5 out of 7) of the
20 cell lines are dispersed over the 445 kb genomic region
on the long arm of chromosome 12 between cRM33 and cRM98.
It is important to note already here that precisely this
region was recently shown to contain the chromosome 12q
breakpoints in cell lines derived from primary uterine
25 leiomyomas (see Fig. 3) and was therefore designated
ULCR12 (Schoenmakers et al., 1994b). As this segment of
the long arm of chromosome 12 is involved in at least two
types of solid tumors (Schoenmakers et al., 1994b; this
study) and, as we will show below, also in a third solid
30 tumor type, we will from now on refer to the DNA interval
between cRM36 and cRM103 as MAR (multiple aberration
region).

FISH Mapping of 12q Breakpoints in Primary Pleomorphic
35 **Salivary Gland Adenomas.**

Our FISH studies on metaphase chromosomes of
pleomorphic adenomas of the salivary glands presented so
far were restricted to cell lines derived from primary

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tumors. Although it is reasonable to assume that the
chromosome 12 breakpoints in cell lines are similar if
not identical to the ones in the corresponding primary
tumors, differences as a result of the establishment of
5   cell lines or subsequent cell culturing cannot fully be
excluded. Therefore, we have investigated whether the
chromosome 12 breakpoints in three primary salivary gland
adenomas were mapping to MAR as well. To test this
possibility, a combination of cosmid clones cRM33 and
10   cRM103 were used as molecular probe. In all three cases,
this cosmid pool clearly spanned the chromosome 12
breakpoints (data not shown), indicating that these
breakpoints were indeed localized within MAR. In a recent
study (Wanschura et al., submitted for publication), it
15   was reported that the chromosome 12 breakpoints of five
primary uterine leiomyomas with 12q14-15 aberrations were
all found to cluster within the 1.5 Mb DNA fragment
(between cRM33 and cRM103), which is known to harbor the
breakpoints of various cell lines derived from primary
20   uterine leiomyomas (schematically summarized in Fig. 3).
Consistent with the results of the breakpoint mapping
studies using cell lines, the results with the two
primary solid tumor types establish that the breakpoints
of the primary tumor cells are located in MAR.
25

Chromosome Segment 12q13-q15 Breakpoints of Lipomas
Mapping within MAR.
        To test the possibility that the chromosome 12
breakpoints of other solid tumors with 12q13-q15
30   aberrations also mapped within MAR, we studied two
lipomas cell lines by FISH analysis---Li-14/SV40 and Li-
538/SV40. The chromosome 12 aberrations of these two
lipoma cell lines are given in Table 1. As molecular
probes, cosmid clones cRM33, cRM53, cRM72, cRM76, cRM99,
35   cRM103, and cRM133 were used. The breakpoint of Li-
14/SV40 was mapped to the 75 kb DNA interval between RM76
and RM133, and that of Li-538/SV40 to the 90 kbp interval
between RM76 and RM99 (data not shown), as schematically

illustrated in Figure 3. Similar FISH analysis of two
primary lipomas using a mixture of cRM36 and cRM103 as
molecular probe resulted in a hybridization pattern
indicating that the mixture of probes detected sequences
5 on either side of the breakpoints. These results are the
first indications that also in lipoma, chromosome 12q13-
q15 breakpoints occur that map within MAR. More lipoma
cases should be tested to allow proper interpretation of
this observation.
10

DISCUSSION

In this study, we have mapped the chromosome 12
breakpoints of three primary pleomorphic salivary gland
15 adenomas as well as seven established cell lines derived
from such tumors. All breakpoints appeared to be located
in a previously molecularly cloned and characterized
chromosome DNA segment on the long arm of chromosome 12,
of about 1.7 Mb in size, with five of them clustering in
20 a DNA interval of less than 500 kb. The 1.7 Mb DNA region
apparently contains a major breakpoint cluster region for
this type of tumor. In a previous study, we have
described the characterization of the chromosome 12
breakpoint of pleomorphic salivary gland adenoma cell
25 line Ad-312/SV40 (Kools et al., 1995). The breakpoint of
this cell line is now known to map at a distance of more
than 2 Mb distally to this major breakpoint cluster
region reported here. It is possible that the Ad-312/SV40
breakpoint involves other pathogenetically relevant
30 genetic sequences than those affected by the clustered
breakpoints. However, the possibility should not yet be
excluded that all the 12q13-q15 breakpoints in
pleomorphic salivary gland adenomas mapped so far belong
to the same category and are dispersed over a relatively
35 large DNA region of this chromosome, reminiscent of the
11q13 breakpoints in B-cell malignancies (Raynaud et al.,
1993). More precise pinpointing of the various
breakpoints could shed more light on this matter.

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Of importance is the observation that the DNA
segment that harbors the clustered 12q breakpoints of
pleomorphic salivary gland adenomas appears to coincide
with the DNA region that was recently defined as the
5 uterine leiomyoma cluster region of chromosome 12
breakpoints, known as ULCR12 (Schoenmakers et al.,
1994b). Of further interest is the fact that this region
of chromosome 12 also harbors breakpoints of primary
lipomas and lipoma cell lines derived from primary tumors
10 with 12q13-q15 aberrations. Altogether, the results of
all these studies now clearly demonstrate that chromosome
12 breakpoints of three distinct solid tumor types map to
the same 1.7 Mb genomic region on the long arm of
chromosome 12, establishing this region to be a multiple
15 aberration region. To reflect this characteristic, we
have designated this DNA segment MAR.

Genetic aberrations involving chromosomal
region 12q13-q15 have been implicated by many cytogenetic
studies in a variety of solid tumors other than the three
20 already mentioned. Involvement of 12q13-q15 has also been
reported for endometrial polyps (Walter et al., 1989;
Vanni et al., 1993), clear cell sarcomas characterized by
recurrent t(12;22)(q13;q13) (Fletcher, 1992; Reeves et
al., 1992; Rodriguez et al., 1992), a subgroup of
25 rhabdomyosarcoma (Roberts et al., 1992) and
hemangiopericytoma (Mandahl et al., 1993a), chondromatous
tumors (Mandahl et al., 1989; Bridge et al., 1992;
Hirabayashi et al., 1992; Mandahl et al., 1993b), and
hamartoma of the lung (Dal Cin et al., 1993). Finally,
30 several case reports of solid tumors with involvement of
chromosome region 12q13-q15 have been published---e.g.,
tumors of the breast (Birdsal et al., 1992; Rohen et al.,
1993), diffuse astrocytomas (Jenkins et al., 1989), and a
giant-cell tumor of the bone (Noguera et al., 1989). On
35 the basis of results of cytogenetic studies, no
predictions could be made about the relative distribution
of the breakpoints of these tumor types. In light of the
results of the present study, it would be of interest to

see whether the breakpoints of any of these solid tumors
also map within or close to MAR. The various cosmid
clones available now provide the means to test this
readily.

5   The observation that 12q breakpoints of at
least three different types of solid tumors map to the
same DNA region is intriguing as it could be pointing
towards the possibility that the same genetic sequences
in MAR are pathogenetically relevant for tumor
10 development in different tissues. If so, it is tempting
to speculate that the gene(s) affected by the genetic
aberrations might be involved in growth regulation. On
the other hand, one cannot yet exclude the possibility
that genetic sequences in MAR are not pathogenetically
15 relevant, as the observed clustering of genetic
aberrations in MAR could simply reflect genetic
instability of this region, which becomes apparent in
various solid tumors. To obtain more insight in this
matter, the genes residing in MAR should be identified
20 and characterized, and this can be achieved by various
approaches using several techniques (Parrish and Nelson,
1993).

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the translocation breakpoints of primary pleomorphic

adenomas and lipomas within a common region of chromosome
12.

LEGENDS OF FIGURES OF ANNEX 1

Figure 1. Schematic representation of FISH mapping data
obtained for the seven pleomorphic salivary gland adenoma
5 cell lines tested in this study. Cosmid clones which were
used as probes in the FISH mapping studies map at
sequence-tagged sites obtained from overlapping YAC
clones. They are named after the acronyms of the STSs, as
shown in the boxes, and the relative order of these is as
10 presented. The DNA interval between RM69 and RM72 is
estimated to be about 2.8 Mb. The solid lines indicate
DNA intervals in which the breakpoints of the various
cell lines are located. The dots indicate FISH
experiments that were performed on metaphase chromosomes
15 of the various cell lines using a cosmid clone
corresponding to the STS indicated above these as
molecular probe. The relative positions of MAR and ULCR12
are indicated in the lower part of the figure. Ad,
pleomorphic salivary gland adenoma; MAR, multiple
20 aberration region; ULCR12, uterine leiomyoma cluster
region of chromosome 12 breakpoints.
Figure 2. a: Partial karyotype of Ad-295/SV40 showing
der(8), der(12), der(18) and the corresponding normal
chromosomes. b: FISH analysis of metaphase chromosomes of
25 Ad-295/SV40 cells using DNA of cosmid clone cRM76 as
molecular probe. Hybridization signals on normal
chromosome 12 (arrow) and der(12) (arrowhead). c: GTG-
banding pattern of metaphase chromosomes of Ad-295/SV40
shown in b. d: FISH analysis of metaphase chromosomes of
30 Ad-295/SV40 cells using DNA of cosmid clone cRM103 as
molecular probe. Hybridization signals on normal
chromosome 12 (arrow) and der(18) (arrowhead).
Figure 3. Schematic representation of chromosome 12
breakpoint mapping data obtained for primary pleomorphic
35 salivary gland adenomas, uterine leiomyomas, and lipomas
as well as cell lines derived from such solid tumors.
Results are compared to data for primary uterine
leiomyomas (Wanschura et al., submitted for publication)

and cell lines derived from such tumors (Schoenmakers et
al., 1994b). Cosmid clones which were used as probes in
the FISH mapping studies correspond to sequence-tagged
sites obtained from overlapping YAC clones. Cosmid clones
5 were named after the acronyms of the STSs, as shown in
the boxes, and the relative order of these is as
presented. The estimated sizes of DNA intervals between
STSs are indicated. Ad, pleomorphic salivary gland
adenoma; Li, lipoma; LM, uterine leiomyoma.

ANNEX 2
LEAD ARTICLE

Identification of the Chromosome 12 Translocation
5 **Breakpoint Region of a Pleomorphic Salivary Gland Adenoma**
with t(1;12)(p22;q15) as the Sole Cytogenetic Abnormality

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Schoenmakers, Jan W.M. Geurts, Raf Mols, Bernd
10 **Kazmierczak, Jörn Bullerdiek, Herman Van den Berghe and**
Wim J.M. Van de Ven

ABSTRACT: Cell line Ad-312/SV40, which was derived from a
primary pleomorphic salivary gland adenoma with
15 t(1;12)(p22;q15), was used in fluorescence in situ
hybridization (FISH) analysis to characterize its
translocation breakpoint region on chromosome 12. Results
of previous studies have indicated that the chromosome 12
breakpoint in Ad-312/SV40 is located proximally to locus
20 D12S8 and distally to the CHOP gene. We here describe two
partially overlapping yeast artificial chromosome (YAC)
clones, Y4854 (500 kbp) and Y9091 (460 kbp), which we
isolated in the context of a chromosome walking project
with D12S8 and CHOP as starting points. Subsequently, we
25 have isolated cosmid clones corresponding to various
sequence-tagged sites (STSs) mapping within the inserts
of these YAC clones. These included cRM51, cRM69, cRM85,
cRM90, cRM91, cRM110, and cRM111.

We present a composite long-range restriction map
encompassing the inserts of these two YAC clones and show
by FISH analysis, that both YACs span the chromosome 12
breakpoint as present in Ad-312/SV40 cells. In FISH

5   studies, cosmid clones cRM85, cRM90 and cRM111 appeared
to map distally to the chromosome 12 breakpoint whereas
cosmid clones cRM51, cRM69, cRM91, and cRM110 were found
to map proximally to it. These results assign the
chromosome 12 breakpoint in Ad-312/SV40 to a DNA region

10 of less than 165 kbp. FISH evaluation of the chromosome
12 breakpoints in five other pleomorphic salivary gland
adenoma cell lines indicated that these are located
proximally to the one in Ad-312/SV40, at a distance of
more than 0.9 Mb from STS RM91. These results, while

15 pinpointing a potentially critical region on chromosome
12, also provide evidence for the possible involvement of
chromosome 12q13-q15 sequences located elsewhere.

INTRODUCTION

20   Pleomorphic salivary gland adenoma constitutes
a benign epithelial tumor that originates from the major
and minor salivary glands. It is the most common type of
salivary gland tumor and accounts for almost 50% of all
neoplasms in these organs; 85% of the tumors are found in

25 the parotid gland, 10% in the minor salivary glands, and
5% in the submandibular gland [1]. About 50% of these
adenomas appear to have a normal karyotype but
cytogenetic studies have also revealed recurrent specific
chromosome anomalies [2, 3]. Frequently observed

30 anomalies include aberrations of chromosome 8, usually
involving the 8q12-q13 region, with the most common
aberration a t(3;8)(p21;q12), and aberrations of
chromosome 12, usually translocations involving region
12q13-q15. Non-recurrent clonal chromosome abnormalities

35 have also been reported. The highly specific pattern of
chromosome rearrangements with consistent breakpoints at
8q12-q13 and 12q13-q15 suggests that these chromosomal
regions harbour genes that might be implicated in the

development of these tumors. Molecular cloning of the
chromosome breakpoints and characterization of their
junction fragments may lead to the identification of
pathogenetically relevant genes. At present, no such
5 molecular data have yet been reported for these tumors.
On the basis of fluorescence in situ
hybridization (FISH) data, the chromosome 12 breakpoints
in six pleomorphic salivary gland adenoma cell lines were
recently shown to be mapping to region 12q13-q15, more
10 precisely, to the genomic interval between loci D12S19
and D12S8 [4, 5]. The sex-averaged genetic size of this
genomic DNA interval was reported at HGM10 to be 7 cM
[6]. We also reported that the chromosome 12 breakpoints
in salivary gland adenomas map distally to the CHOP gene
15 [5], which supports an earlier study indicating that the
12q13-q15 translocation breakpoints in pleomorphic
salivary gland adenomas are different from that in myxoid
liposarcoma [7]. Here, we report about the physical
mapping of the chromosome 12 breakpoint in pleomorphic
20 salivary gland adenoma cell line Ad-312/SV40, which
carries a t(1;12)(p22;q15) as the only cytogenetic
abnormality.

MATERIALS AND METHODS
25 **Tumor Cell Lines.**
Human tumor cell lines used in this study
included the previously described pleomorphic salivary
gland adenoma cell lines Ad-248/SV40, Ad-263/SV40, Ad-
295/SV40, Ad-302/SV40, Ad-312/SV40, and Ad-366/SV40 [5,
30 8]. Cells were cultivated in TC199 culture medium with
Earle's salts supplemented with 20% fetal bovine serum.
Other cell lines used in this study included somatic cell
hybrid PK89-12, which contains chromosome 12 as the sole
human chromosome in a hamster genetic background [9], and
35 somatic cell hybrid LIS-3/SV40/A9-B4 [4]. The latter cell
line was obtained upon fusion of myxoid liposarcoma cell
line LIS-3/SV40, which carries the specific
t(12;16)(q13;p11.2), with mouse A9 cells. This somatic

cell hybrid was previously shown to contain der(16) but
neither der(12) nor the normal chromosome 12 [4]. PK89-12
and LIS-3/SV40/A9-B4 cells were grown in DME-F12 medium
supplemented with 10% fetal bovine serum. Cell lines were
5 analyzed by standard cytogenetic techniques at regular
intervals.

Isolation of YAC and Cosmid Clones.

            In the context of human genome mapping studies,
10 which will be described in detail elsewhere (Schoenmakers
et al., in preparation), we isolated YAC clones Y4854 and
Y9091 from the first-generation CEPH YAC library [10],
and cosmid clones cRM51, cRM69, cRM85, cRM90, cRM91,
cRM103, cRM110, and cRM111 from the chromosome-12-
15 specific, arrayed cosmid library LLNLNC01 [11]. YAC and
cosmid clones were isolated as described before [5].
Initial screenings of the YAC, as well as the cosmid
library, were performed using a screening strategy
involving the polymerase chain reaction (PCR) [12].
20 Filter hybridization analysis was used as the final
screening step, as previously described [5]. Cosmid
clones were isolated using STSs and those corresponding
to STSs within the inserts of YAC clones Y4854 and Y9091
are indicated in Fig. 1. STSs were obtained via rescue of
25 YAC insert end-sequences using a vectorette-PCR procedure
[13] or Alu-PCR [14, 15]. PCR products were sequenced
directly via solid-phase fluorescent sequencing. Cosmid
clones were grown and handled according to standard
procedures [16]. YAC clones were characterized by pulsed-
30 field gel electrophoresis [17], restriction mapping, and
hybridization, as previously described [5].

**Chromosome Preparations and Fluorescence in Situ
Hybridization.**

35   Cells from the pleomorphic salivary gland
adenoma tumor cell lines were treated with Colcemid (0.04
µg/ml) for 30 min and then harvested according to routine
methods. Metaphase spreads of the tumor cells were

prepared as described before [4]. To establish the
identity of chromosomes in the FISH experiments, FISH
analysis was performed after G-banding of the same
metaphase spreads. G-banding was performed essentially as
5 described by Smit et al. [18]. In situ hybridizations
were carried out according to a protocol described by
Kievits et al. [19] with some minor modifications [5,
20]. Cosmid and YAC DNA was labelled with biotin-11-dUTP
(Boehringer Mannheim) or biotin-14-dATP (BRL,
10 Gaithersburg), as described earlier [5]. Chromosomes were
counterstained with propidium iodide and analyzed on a
Zeiss Axiophot fluorescence microscope using a FITC
filter (Zeiss). Results were recorded on Scotch (3M)
640asa film.

15

RESULTS
**Isolation and Characterization of YAC Clones Spanning the
Chromosome 12 Breakpoint of Pleomorphic Salivary Gland
Adenoma Cell Line Ad-312/SV40.**
20   In previous studies [5], we mapped the
chromosome 12 breakpoints of six pleomorphic salivary
gland adenoma cell lines proximally to locus D12S8 and
distally to CHOP. The DNA interval between these loci is
somewhat smaller than 7 cM (estimated distance between
25 the loci D12S8 and D12S19 [6]) but still substantially
large. To molecularly define the translocation breakpoint
of Ad-312/SV40, we have performed human genome mapping
studies on the DNA interval between locus D12S8 and the
CHOP gene. In the process of directional chromosome
30 walking starting from D12S8 and the CHOP gene, we
obtained overlapping YAC clones Y9091 and Y4854. The DNA
insert of Y9091 appeared to be 460 kbp and that of Y4854,
500 kbp. Moreover, as we will demonstrate below, the DNA
insert of each YAC clone appeared to span the chromosome
35 12 breakpoint of Ad-312/SV40. A long-range restriction
map of the inserts of these YAC clones was made using
pulsed-field gel electrophoresis and hybridization
analysis (Fig. 1). On the basis of STS content mapping

and Southern blot analysis, the inserts of YAC clones
Y9091 and Y4854 appeared to overlap as indicated in
Figure 1. The tested STSs correspond to end-sequences of
other overlapping YAC clones not shown here or to
5 sequences obtained via inter-Alu-PCR. Of these, RM90 and
RM91 represent such end-clone STSs of YAC Y9091, and RM48
and RM54 of Y4854, whereas RM110 and RM111 represent STSs
derived from inter-Alu-PCR. For a number of STSs mapping
within the inserts of YAC clones Y4854 and Y9091,
10 corresponding cosmid clones were isolated for use in FISH
analysis, e.g., cRM51, cRM69, cRM85, cRM90, cRM91,
cRM110, and cRM111.

      The inserts of the two overlapping YAC clones
are most likely not chimeric, as was deduced from the
15 following observations. FISH analysis of metaphase
chromosomes of normal human lymphocytes with Y4854 or
Y9091 DNA as molecular probe revealed hybridization
signals only in chromosome region 12q13-q15. For Y9091,
this was confirmed further by observations made in FISH
20 studies in which cosmid clone cRM90 or cRM91 was used as
probe; the DNA insert of each of these two cosmids
corresponds to the alternative end-sequences of YAC clone
Y9091. Finally, the end-sequence STSs of Y9091 appeared
to map to chromosome 12 and distally to the CHOP gene, as
25 was established by PCR analysis on PK89-12 DNA, which
contains human chromosome 12 as the sole human chromosome
in a hamster genetic background, and LIS-3/SV40/A9-B4
DNA, which was previously shown to contain der(16), from
the specific t(12;16) of myxoid liposarcoma, but neither
30 der(12) nor the normal chromosome 12 [4]. From the
chromosome walking studies, we concluded that the
overlapping inserts of the two YAC clones represent a DNA
region of about 640-kbp, which is located on chromosome
12q between D12S8 and CHOP. As the 640-kbp composite
35 long-range restriction map of the YAC contig was
constructed with at least double coverage of the entire
region, it is not unreasonable to assume that the 640-kbp

region is contiguous with the chromosomal DNA, although
microdeletions can not be excluded at this point.

Chromosome walking was routinely evaluated by
FISH mapping of YAC clones and/or cosmid clones
5   corresponding to YAC insert sequences. It should be noted
that for the identification of chromosomes, G-banding was
used in most cases. On the basis of such G-banding,
hybridization signals could be assigned conclusively to
chromosomes of known identity; this was also of
10   importance for the cases with cross- or background
hybridization signals that were occasionally observed. G-
banding prior to FISH analysis resulted sometimes in
rather weak hybridization signals or rather vague banding
patterns. Therefore, we performed FISH experiments in
15   which the YAC and cosmid clones to be evaluated were used
in combination with a reference probe. Cosmid clone
cPK12qter, which was serendipitously obtained during
screening of a cosmid library, was selected as reference
marker. FISH analysis of metaphase chromosomes of normal
20   lymphocytes (Fig. 2A) revealed that cPK12qter maps to the
telomeric region of the long arm of chromosome 12. To
identify chromosome 12 in this experiment, centromere 12-
specific probe pα12H8 [21] was used. FISH analysis of
metaphase chromosomes of Ad-312/SV40 cells using YAC
25   clone Y4854 (Fig. 2B) or Y9091 (Fig. 2C) in combination
with reference probe cPK12qter revealed, in both cases,
hybridization signals of the YAC insert on der(1) as well
as der(12). We concluded from these results that the
insert DNA of each YAC clone might span the chromosome 12
30   breakpoint in this cell line. It should be noted that G-
banding revealed a telomeric association involving the
short arm of chromosome 12 in Figure 2C. The observation
that YAC clone Y9091 spanned the chromosome 12 breakpoint
in Ad-312/SV40 was confirmed independently in FISH
35   studies in which cosmid clone cRM90 or cRM91 was used as
molecular probe; they were shown to contain the
alternative end-sequences of the Y9091 insert. cRM90
appeared to map distally to the chromosome 12 breakpoint,

whereas cRM91 was found to map proximally (data not
shown). These results also established the chromosomal
orientation of the YAC contig shown in Figure 1. In
summary, we concluded from these FISH studies that the
5   chromosome 12 translocation breakpoint in Ad-312/SV40
must be located in the DNA interval corresponding to the
overlapping sequences (about 300 kbp) of the two YAC
clones.

10 **Fine Mapping of the Chromosome 12 Translocation
Breakpoint of Ad-312/SV40.**

In an approach to further narrow the chromosome
12 translocation breakpoint region of Ad-312/SV40, cosmid
clones with different mapping positions within YAC clone
15 Y9091 were isolated. These included cRM69, cRM85, cRM110,
and cRM111. cRM69 and cRM85 were isolated on the basis of
STS sequences of YAC clones not shown here. cRM110 and
cRM111 were obtained via inter-Alu-PCR. RM110 was shown
by Southern blot analysis to hybridize to a terminal MluI
20 fragment of Y9091 and not to the DNA insert of the
overlapping YAC clone with RM69 as telomeric end-
sequences. The location of RM110 is as indicated in
Figure 1. RM111 was shown to hybridize to a BssHII, MluI,
PvuI, and SfiI fragment of Y9091 and is therefore located
25 in the PvuI-SfiI fragment of Y9091, to which STS RM48 was
also mapped (Fig. 1). FISH analysis of metaphase
chromosomes of Ad-312/SV40 with cRM69 or cRM110 as probe
indicated that the DNA insert of these cosmids mapped
proximally to the chromosome 12 translocation breakpoint
30 in this cell line, as illustrated for cRM69 in Figure 3A.
Subsequent FISH analysis of Ad-312/SV40 with cRM85 or
cRM111 as probe revealed hybridization signals distally
to the translocation breakpoint, as illustrated for
cRM111 in Figure 3B. The results with cRM85 and cRM111
35 are in agreement with the observed breakpoint spanning by
YAC clone Y4854 as cRM85 maps distally and cRM111 closely
to STS RM48, which marks the telomeric end of YAC clone
Y4854. In conclusion, the chromosome 12 translocation

breakpoint in Ad-312/SV40 must be located in the DNA
interval between cRM110 and cRM111, as schematically
summarized in Figure 4.

5 **FISH Evaluation of Chromosome 12 Breakpoints in Other
Pleomorphic Salivary Gland Adenoma Cell Lines.**

To determine the position of their chromosome
12 breakpoints relative to that of Ad-312/SV40, five
other pleomorphic salivary gland adenoma cell lines were
10 evaluated by FISH analysis, as summarized schematically
in Figure 4. These cell lines, which were developed from
primary tumors [5, 8], included Ad-248/SV40, Ad-263/SV40,
Ad-295/SV40, Ad-302/SV40, and Ad-366/SV40. The chromosome
12 aberrations of these cell lines are listed in Figure
15 4. FISH analysis of metaphase chromosomes of these cell
lines using cRM91 revealed that the chromosome 12
breakpoints of all these cell lines mapped proximally to
this cosmid clone (data not shown). Similar FISH analysis
was also performed using a cosmid clone corresponding to
20 sequence-tagged site RM103 as a probe. RM103 was found to
map proximally to RM91 at a distance of about 0.9 Mbp. In
all cases, cRM103 appeared to map distally to the
chromosome 12 translocation breakpoints, indicating that
the chromosome 12 breakpoints in these five pleomorphic
25 salivary gland adenoma cell lines are located at a
relatively large distance from that of Ad-312/SV40 cells.

DISCUSSION

In the studies presented here, we have
30 identified, molecularly cloned, and characterized a
chromosome region on the long arm of chromosome 12 in
which the translocation breakpoint of pleomorphic
salivary gland adenoma cell line Ad-312/SV40 appears to
map. In previous studies [5], we already provided
35 evidence that the chromosome 12 breakpoint of this cell
line was located between D12S8 and CHOP. Because the two
breakpoints spanning YAC clones described here were
obtained in directional chromosome walking experiments

using D12S8 and the CHOP gene as initial starting points,
the chromosome 12 breakpoint mapping results presented
here confirm our previous claim. The FISH results
obtained with the complete YAC insert of Y9091 as
5   molecular probe were confirmed independently in FISH
studies using cosmid clones containing sequences
corresponding to various regions of the insert of this
YAC clone. This is of importance, as the independent
confirmatory results make it rather unlikely that the
10 split signals observed with the complete insert of Y9091
can be explained otherwise than by a factual splitting of
sequences represented in the YAC. The presence, for
instance, of highly related genetic sequences on both
sides of a chromosome breakpoint could easily lead to
15 erroneous conclusions if they were based solely on FISH
results of a YAC insert. Finally, our mapping studies
have also established conclusively the chromosomal
orientation of the long-range restriction map we have
generated in these studies. This orientation was already
20 predicted on the basis of two-color FISH studies
(unpublished observations).

        The FISH studies, described here, enabled us to
map the chromosome 12 breakpoint in Ad-312/SV40 cells to
the 190-kbp DNA interval between the established STSs
25 RM48 and RM69. However, the breakpoint region can be
narrowed somewhat further on the basis of the following.
The fact that Y4854 was shown to span the breakpoint
indicates that at least a considerable part of the
telomeric half of this YAC clone must map distally to the
30 breakpoint. Precisely how much remains to be established.
On the other side, STS RM69 appeared to be located in
about the middle of the DNA insert of cosmid clone cRM69,
suggesting that the breakpoint is close to 25 kbp
distally to RM69. Moreover, cRM69 appeared to lack RM110
35 (data not shown) and, as cRM110 was found proximally to
the chromosome 12 breakpoint in Ad-312/SV40 cells, the
breakpoint should be even further distal to RM69 than the
earlier-mentioned 25 kbp. Altogether, this narrows the

chromosome 12 breakpoint region to a DNA interval, which
must be considerably smaller than 165 kbp. Further
pinpointing of the breakpoint will allow us to
molecularly clone the chromosome 12 breakpoint and to
5 characterize the genetic sequences in the breakpoint
junction region, which might lead to the identification
of pathogenetically relevant sequences. Identification of
the genes present in the DNA inserts of YAC clones Y4854
and Y9091, via sequencing, direct hybridization, direct
10 selection or exon-trapping, might constitute a useful
alternative approach for identifying the gene in this
region of the long arm of chromosome 12 that might be
pathogenetically critical for pleomorphic salivary gland
adenoma tumorigenesis.
15   The observation that the chromosome 12
breakpoints in other pleomorphic salivary gland adenomas
are located in a remote and more proximal region on the
long arm of chromosome 12 is of interest. It could imply
that the chromosome 12 breakpoints in pleomorphic
20 salivary gland adenomas are dispersed over a relatively
large DNA region of the long arm of chromosome 12,
reminiscent to the 11q13 breakpoints in B-cell
malignancies [22]. Elucidation of the precise location of
the chromosome 12 breakpoints in the other pleomorphic
25 salivary gland adenoma cell lines could shed more light
on this matter. On the other hand, it could point towards
alternative sequences on the long arm of chromosome 12
between D12S8 and the CHOP gene that might be of
importance, presumably for growth regulation in
30 pleomorphic salivary gland adenoma. The fact that the
chromosome 12 breakpoint region described here has sofar
been found only in the Ad-312/SV40 cell line makes it
necessary to analyze a larger number of salivary gland
adenomas with chromosome 12q13-q15 aberrations to assess
35 the potential relevance for tumorigenesis of the
chromosome 12 sequences affected in the studied cell
line. If more cases with aberrations in this particular
region of chromosome 12 can be found, it would be of

interest to find out whether these tumors form a clinical
subgroup. Finally, chromosome translocations involving
region q13-q15 of human chromosome 12 have been reported
for a variety of other solid tumors: benign adipose
5 tissue tumors, uterine leiomyoma, rhabdomyosarcoma,
hemangiopericytoma, clear-cell sarcoma, chondromatous
tumors, and hamartoma of the lung. Whether or not the
chromosome 12 breakpoints in some of these tumors map
within the same region as that of Ad-312/SV40 remains to
10 be established. The YAC and cosmid clones described in
this report constitute useful tools to investigate this.

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LEGENDS OF FIGURES OF ANNEX 2

Figure 1. Composite physical map of the overlapping DNA
inserts of YAC clones Y4854 and Y9091. Sizes of the DNA
inserts are indicated. The relative positions of the YAC
5 clones are represented by bars below the long range
physical map. Sequence-tagged sites (STSs) corresponding
to end-clones of YACs, including YACs not shown here, are
indicated by boxed RM codes above the restriction map.
STSs obtained from inter-Alu-PCR products are given below
10 the restriction map and the DNA regions to which they
have been mapped are marked by arrows. B: BssHII; M:
MluI; P: PvuI; Sf: SfiI. A polymorphic MluI site is
marked by an asterisk.

Figure 2. A) Mapping of cosmid clone cPK12qter to the
15 telomeric region of the long arm of chromosome 12.
Centromere 12-specific probe pα12H8 was used to establish
the identity of chromosome 12. FISH analysis was
performed on metaphase chromosomes of control human
lymphocytes. Hybridization signals of cPK12qter are
20 marked with small arrowheads, those of the centromere 12-
specific probe with asterisks. B, C) FISH analysis of
metaphase chromosomes of Ad-312/SV40 cells using DNA of
YAC clone Y4854 (B) or Y9091 (C) as molecular probe in
combination with cosmid clone cPK12qter as reference
25 marker. Hybridization signals of the YAC clones on
chromosome 12 are indicated by large arrowheads; those on
der(1) by large arrows, and those on der(12) by small
arrows, respectively. The hybridization signals of cosmid
clone cPK12qter are indicated by small arrowheads.

30 **Figure 3.** FISH analysis of metaphase chromosomes of Ad-
312/SV40 cells using DNA of cosmid clone cRM69 (A) or
cRM111 (B) as molecular probe in combination with cosmid
clone cPK12qter as reference marker. The position of the
hybridization signals of cPK12qter are indicated by small
35 arrowheads. In (A), the position of the hybridization
signal of cRM69 on normal chromosome 12 is indicated by a
large arrowhead, and that on der(12) with a small arrow.
In (B), the position of the hybridization signal of

cRM111 on normal chromosome 12 is indicated by a large
arrowhead, and that on der(1) with a large arrow.

Figure 4. Schematic representation of FISH mapping data
obtained for the six pleomorphic salivary gland adenoma

5 cell lines tested in this study. The specific chromosome
12 aberrations in the various cell lines are given.
Cosmid clones which were used as probes in the FISH
mapping studies correspond to sequence-tagged sites
obtained from overlapping YAC clones. Individual FISH

10 experiments are indicated by dots. Cosmid clones were
named after the acronyms of the STSs, as shown in the
boxes, and the relative order of these is as presented.
The DNA interval between RM90 and RM103 is estimated to
be about 1.3 Mb. Insert: Schematic representation of the

15 G-banded derivative chromosomes der(1) and der(12) of the
Ad-312/SV40 cell line, which carries a t(1;12)(p22;q15).
The positions of the chromosome 12 breakpoints of Ad-
248/SV40, Ad-263/SV40, Ad-295/SV40, Ad-302/SV40, and Ad-
366/SV40 are distal to RM103 as indicated by the arrow.

CLAIMS

1. Multi-tumor Aberrant Growth (MAG) gene having the nucleotide sequence of any one of the strands of any one of the members of the High Mobility Group protein genes or LIM protein genes, including modified versions thereof.

5   2. Multi-tumor Aberrant Growth (MAG) gene as claimed in claim 1 having essentially the nucleotide sequence of the HMGI-C gene as depicted in figure 7, or the complementary strand thereof, including modified or elongated versions of both strands.

10   3. Multi-tumor Aberrant Growth (MAG) gene as claimed in claim 1 having essentially the nucleotide sequence of the LPP gene as depicted in figure 5, or the complementary strand thereof, including modified or elongated versions of both strands.

15   4. Multi-tumor Aberrant Growth (MAG) gene as claimed in claim 1, 2 or 3 for use as a starting point for designing suitable expression-modulating compounds or techniques for the treatment of non-physiological proliferation phenomena in human or animal.

20   5. Multi-tumor Aberrant Growth (MAG) gene as claimed in claim 1, 2 or 3 for use as a starting point for designing suitable nucleotide probes for (clinically/medically) diagnosing cells having a non-physiological proliferative capacity as compared to wildtype
25 cells.

6. Protein encoded by the Multi-tumor Aberrant Growth (MAG) gene as claimed in claim 1, 2 or 3 for use as a starting point for preparing suitable antibodies for (clinically/medically) diagnosing cells having a non-
30 physiological proliferative capacity as compared to wildtype cells.

7. Derivatives of the MAG gene as claimed in claim 1, 2 or 3 or of its immediate vicinity for use in diagnosis and the preparation of therapeutical compositions, wherein
35 the derivatives are selected from the group consisting of sense and anti-sense cDNA or fragments thereof, transcripts

of the gene or practically usable fragments thereof,
antisense RNA, fragments of the gene or its complementary
strand, proteins encoded by the gene or fragments thereof,
antibodies directed to the gene, the cDNA, the transcript,
5 the protein or the fragments thereof, as well as antibody
fragments.

8. In situ diagnostic method for diagnosing cells
having a non-physiological proliferative capacity,
comprising at least some of the following steps:
10   a) designing a set of nucleotide probes based on
the information obtainable from the nucleotide sequence of
the MAG gene as claimed in claim 1 or 2, wherein one of the
probes is hybridisable to a region of the aberrant gene
substantially mapping at the same locus as a corresponding
15 region of the wildtype gene and the other probe is
hybridisable to a region of the aberrant gene mapping at a
different locus than a corresponding region of the wildtype
gene;

        b) incubating one or more interphase or metaphase
20 chromosomes or cells having a non-physiological
proliferative capacity, with the probe under hybridising
conditions; and

        c) visualising the hybridisation between the probe
and the gene.
25   9. Method of diagnosing cells having a non-
physiological proliferative capacity, comprising at least
some of the following steps:

        a) taking a biopsy of the cells to be diagnosed;

        b) isolating a suitable MAG gene-related
30 macromolecule therefrom;

        c) analysing the macromolecule thus obtained by
comparison with a wildtype reference molecule preferably
from the same individual.

        10. Method as claimed in claim 9, comprising at
35 least some of the following steps:

        a) taking a biopsy of the cells to be diagnosed;

        b) extracting total RNA thereof;

c) preparing at least one first strand cDNA of the mRNA species in the total RNA extract, which cDNA comprises a suitable tail;

d) performing a PCR and/or RT-PCR using a MAG gene specific primer and a tail-specific and/or partner-specific/nested primer in order to amplify MAG gene specific cDNA's;

e) separating the PCR products on a gel to obtain a pattern of bands;

f) evaluating the presence of aberrant bands by comparison to wildtype bands, preferably originating from the same individual.

11. Method as claimed in claim 9, comprising at least some of the following steps:

a) taking a biopsy of the cells to be diagnosed;

b) isolating total protein therefrom;

c) separating the total protein on a gel to obtain essentially individual bands and optionally trnasferring the bands to a Western blot;

d) hybridising the bands thus obtained with antibodies directed against a part of the protein encoded by the remaining part of the MAG gene and against a part of the protein encoded by the substitution part of the MAG gene;

e) visualising the antigen-antibody reactions and establishing the presence of aberrant bands by comparison with bands from wildtype proteins, preferably originating from the same individual. .

12. Method as claimed in claim 9, comprising at least some of the following steps:

a) taking a biopsy of the cells to be diagnosed;

b) isolating total DNA therefrom;

c) digesting the DNA with one or more so-called "rare cutter" restriction enzymes;

d) separating the digest thus prepared on a gel to obtain a separation pattern;

e) optionally transfering the separation pattern to a Southern blot;

f) hybridising the separation pattern in the gel
or on the blot with one or more informative probes under
hybridising conditions;

g) visualising the hybridisations and establishing
5   the presence of aberrant bands by comparison to wildtype
bands, preferably originating from the same individual.

13. Method as claimed in claim 9, comprising at
least some of the following steps:

a) taking a biopsy of the cells to be diagnosed;
10   b) extracting mRNA therefrom;

c) establishing the presence or the (relative)
quantity of mRNA derived from the MAG gene; and

d) comparing the result of step c) with the result
of a similar experiment with wildtype cells, preferably
15 originating from the same individual.

14. Method as claimed in any one of the claims 8-
13, wherein the cells having a non-physiological
proliferative capacity are selected from the group
consisting of the mesenchymal tumors hamartomas (e.g. breast
20 and lung), adipose tissue tumors (e.g. lipomas), pleomorphic
salivary gland adenomas, uterine leiomyomas, angiomyxomas,
fibroadenomas of the breast, polyps of the endometrium,
atherosclerotic plaques, and other benign tumors as well as
various malignant tumors, including but not limited to
25 sarcomas (e.g. rhabdomyosarcoma, osteosarcoma) and
carcinomas (e.g. of breast, lung, skin, thyroid), and
haematological malignancies, like leukemias and lymphomas.

15. Anti-sense molecules of a MAG gene as claimed
in claim 1, 2 or 3 for use in the treatment of diseases
30 involving cells having a non-physiological proliferative
capacity by modulating the expression of the gene.

16. Expression modulators, such as inhibitors or
enhancers, including ribozymes, of the MAG gene as claimed
in claim 1, 2 or 3 for use in the treatment of diseases
35 involving cells having a non-physiological proliferative
capacity.

17. Antisense RNA molecules complementary to the
mRNA molecules of the MAG gene and/or antibodies directed

against the gene product of the MAG gene as claimed in claim
1, 2 or 3 for use in the treatment of diseases involving
cells having a non-physiological proliferative capacity.

18. Diagnostic kit for performing the method as
5 claimed in claim 8, comprising a suitable set of labeled
nucleotide probes.

19. Diagnostic kit for performing the method as
claimed in claim 10, comprising a suitable set of labeled
probes.

10 20. Diagnostic kit for performing the method as
claimed in claim 11, comprising a suitable set of labeled
MAG gene specific and tail specific PCR primers.

21. Diagnostic kit for performing the method as
claimed in claim 11, comprising a suitable set of labeled
15 probes, and suitable rare cutting restriction enzymes.

22. Pharmaceutical composition for lowering the
expression level of the MAG gene in cells having a non-
physiological proliferative capacity, comprising one or more
of the derivatives as claimed in claim 7 and/or one or more
20 expression modulators as claimed in claim 16.

23. Pharmaceutical composition as claimed in claim
22, wherein the cells having a non-physiological
proliferative capacity are selected from the group
consisting of the mesenchymal tumors hamartomas (e.g. breast
25 and lung), adipose tissue tumors (e.g. lipomas), pleomorphic
salivary gland adenomas, uterine leiomyomas, angiomyxomas,
fibroadenomas of the breast, polyps of the endometrium,
atherosclerotic plaques, and other benign tumors as well as
various malignant tumors, including but not limited to
30 sarcomas (e.g. rhabdomyosarcoma, osteosarcoma) and
carcinomas (e.g. of breast, lung, skin, thyroid), and
haematological malignancies, like leukemias and lymphomas.

24. Use of the derivatives as claimed in claim 7
for the preparation of a diagnostic kit or a pharmaceutical
35 composition for the diagnosis or treatment of diseases or
disorders involving cells having a non-physiological
proliferative capacity.

25. Use of the expression modulators as claimed in claim 16 for the preparation of a pharmaceutical composition for the treatment of diseases or disorders involving cells having a non-physiological proliferative capacity.

5   26. Use as claimed in claim 24 or 25, wherein the cells having a non-physiological proliferative capacity are selected from the group consisting of the mesenchymal tumors hamartomas (e.g. breast and lung), adipose tissue tumors (e.g. lipomas), pleomorphic salivary gland adenomas, uterine

10 leiomyomas, angiomyxomas, fibroadenomas of the breast, polyps of the endometrium, atherosclerotic plaques, and other benign tumors as well as various malignant tumors, including but not limited to sarcomas (e.g. rhabdomyosarcoma, osteosarcoma) and carcinomas (e.g. of

15 breast, lung, skin, thyroid), and haematological malignancies, like leukemias and lymphomas.

27. Method for isolating other MAG genes based on the existence of a fusion gene, fusion transcript or fusion protein in a tumor cell by using at least a part of a MAG

20 gene for designing molecular tools (probes, primers etc.).

28. MAG genes obtainable by the method of claim 27.

29. MAG genes as claimed in claim 28 for use in diagnostic or therapeutic methods.

25   30. Animal model for the assessment of the utility of compounds or compositions in the treatment of diseases or disorders involving cells having a non-physiological proliferative capacity, which animal is a transgenic animal harbouring a MAG gene in its genome.

30   31. Animal model as claimed in claim 30, wherein the MAG gene is an aberrant MAG gene, such as a fusion product of the remaining part of the gene and the substitution part of its translocation partner.

32. Animal model as claimed in claim 30, wherein

35 the MAG gene shows a non-physiological expression level.

33. Animal model for the assessment of the utility of compounds or compositions in the treatment of diseases or disorders involving cells having a non-physiological

proliferative capacity, which animal harbours a specific
genetic aberration affecting a MAG gene as claimed in claim
1, 2 or 3 in the genome of at least part of its cells, which
aberration is induced via homologous recombination in
5 embryonic stem cells.

        34. Animal model as claimed in any one of the
claims 30-33, which animal is a mammal, in particular a
mouse, rat, dog, pig or higher primate, like chimpanzee.

        35. Poly- or oligonucleotide probes and primers as
10 disclosed in the description and figures.
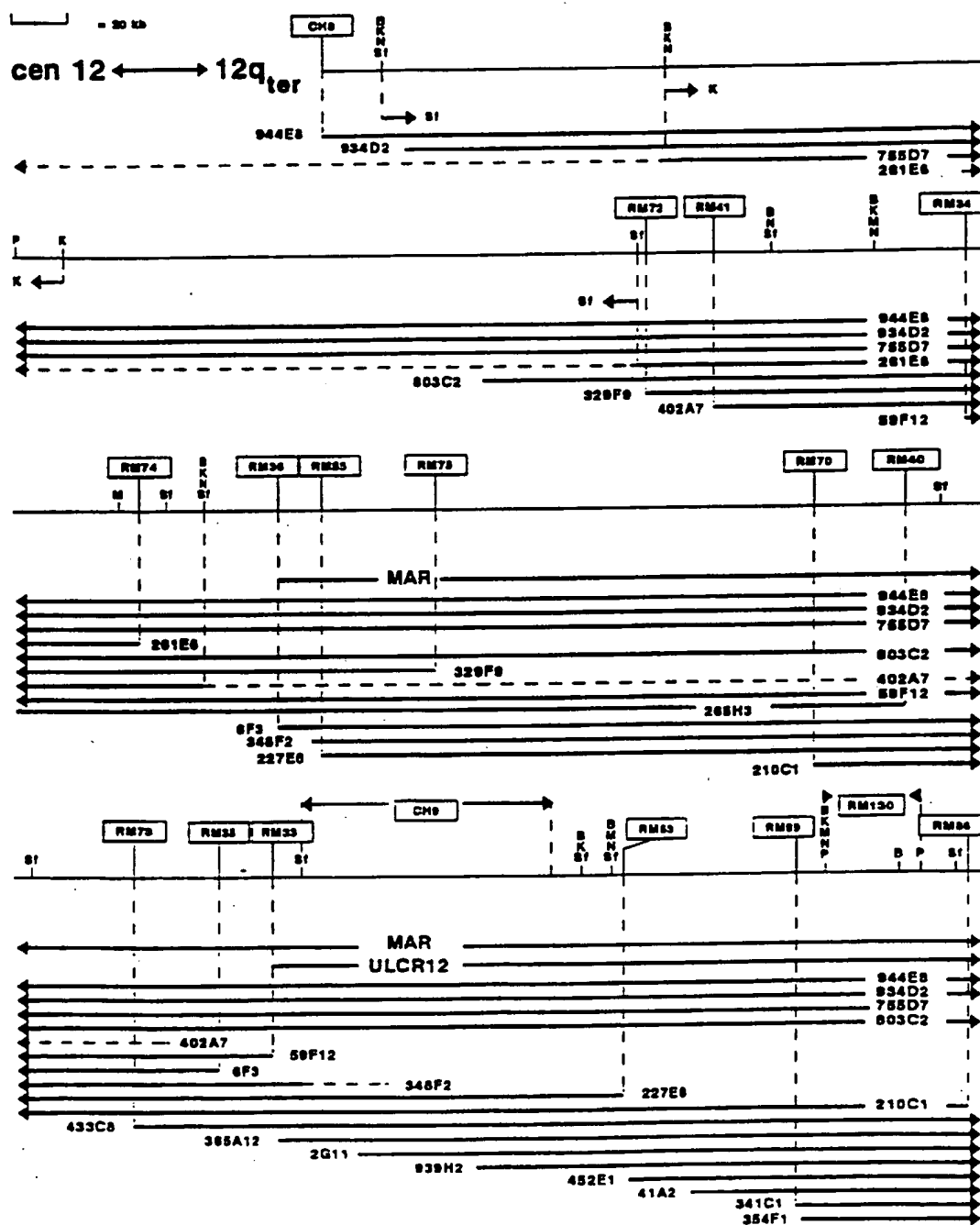
FIG. 1-1
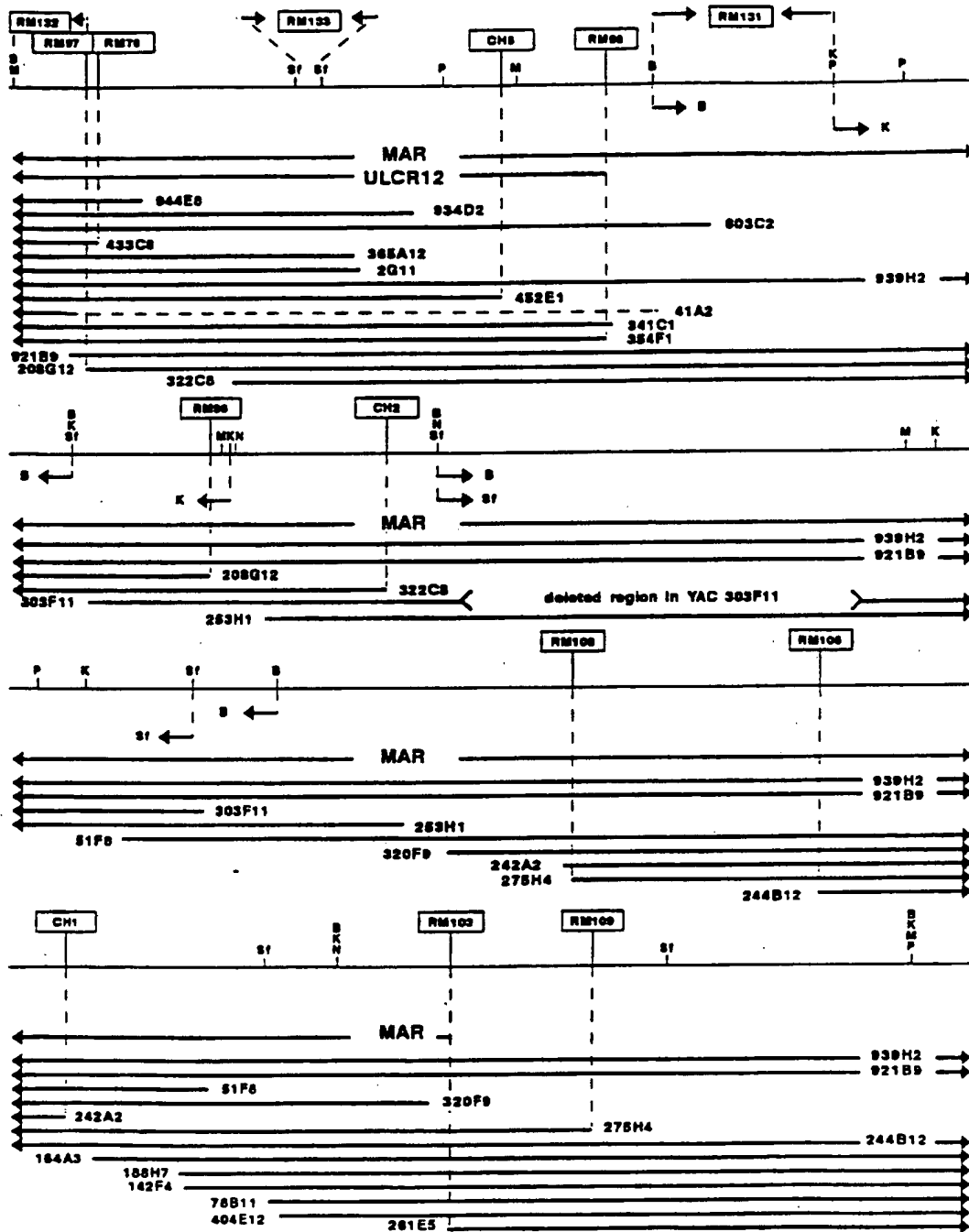
FIG. 1-2
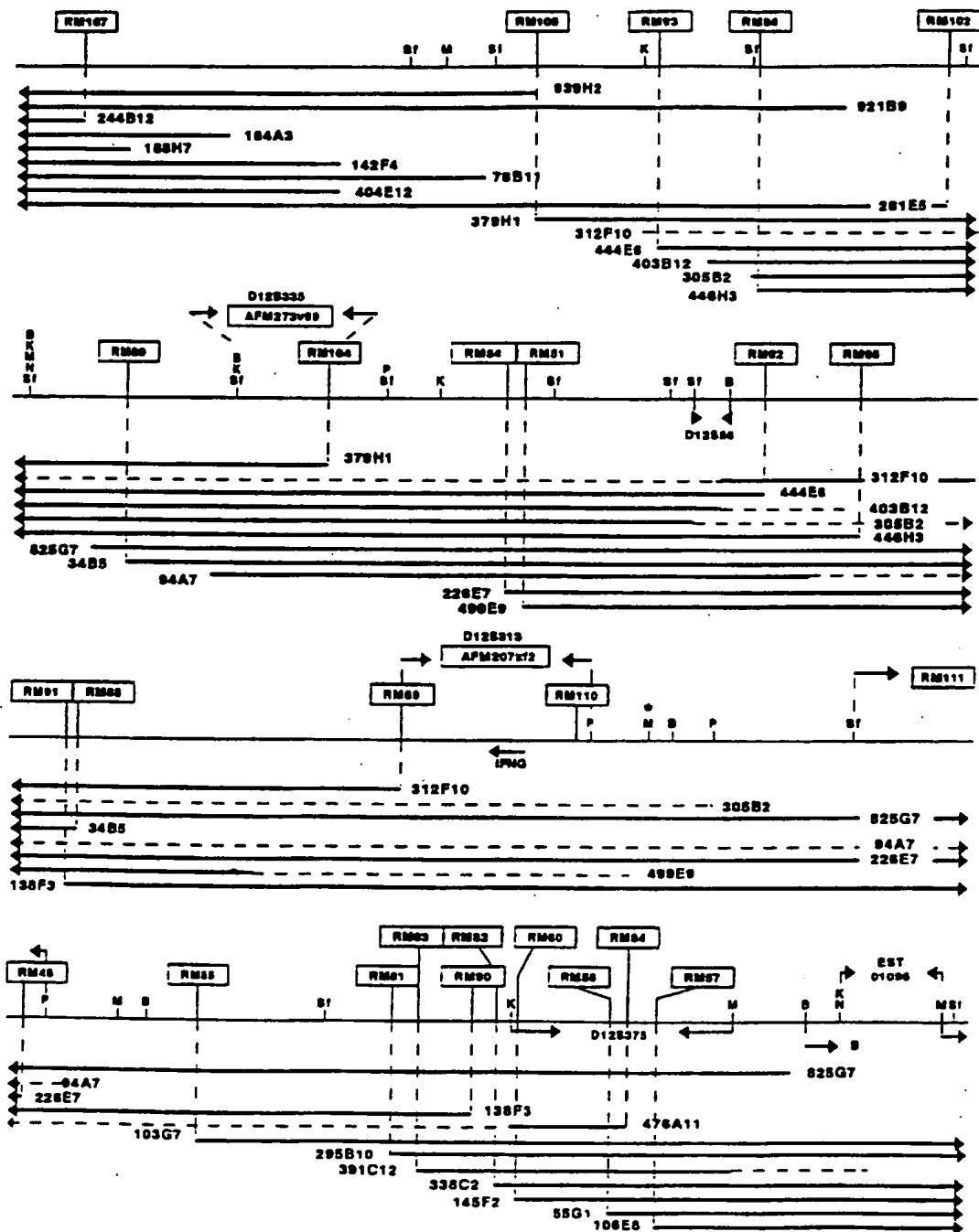
FIG. 1-3
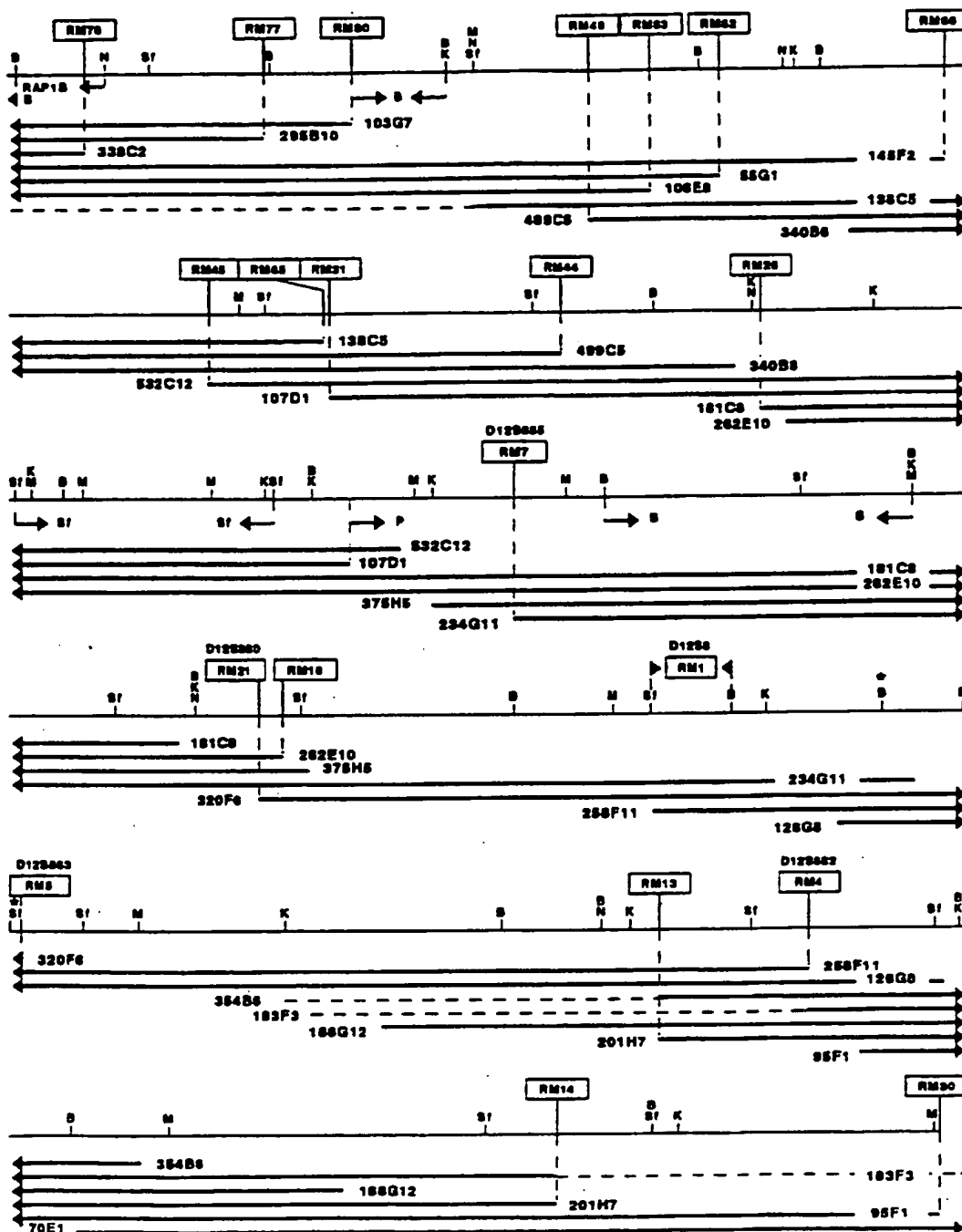
FIG. 1-4
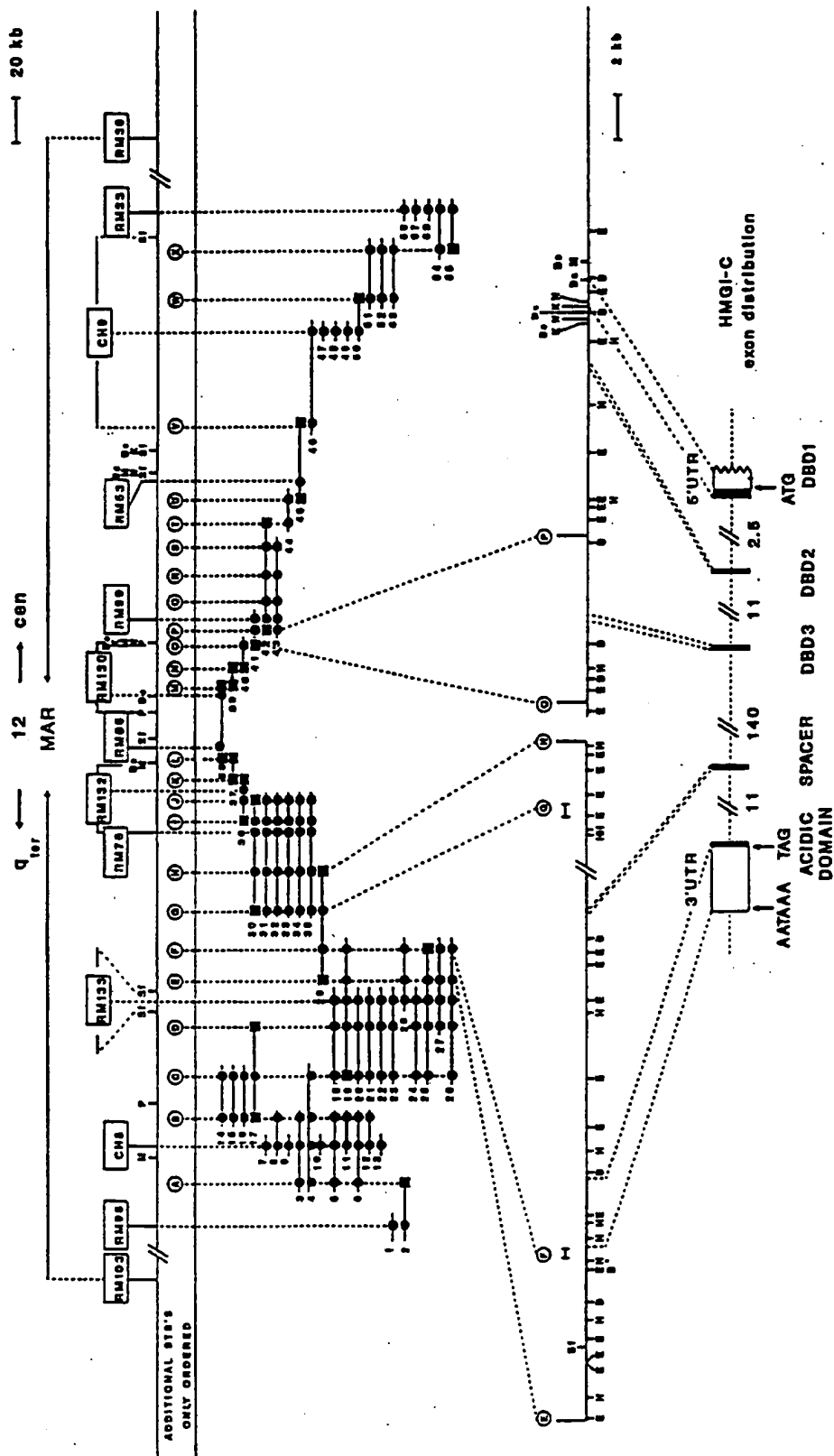
FIG.2

FIG.3

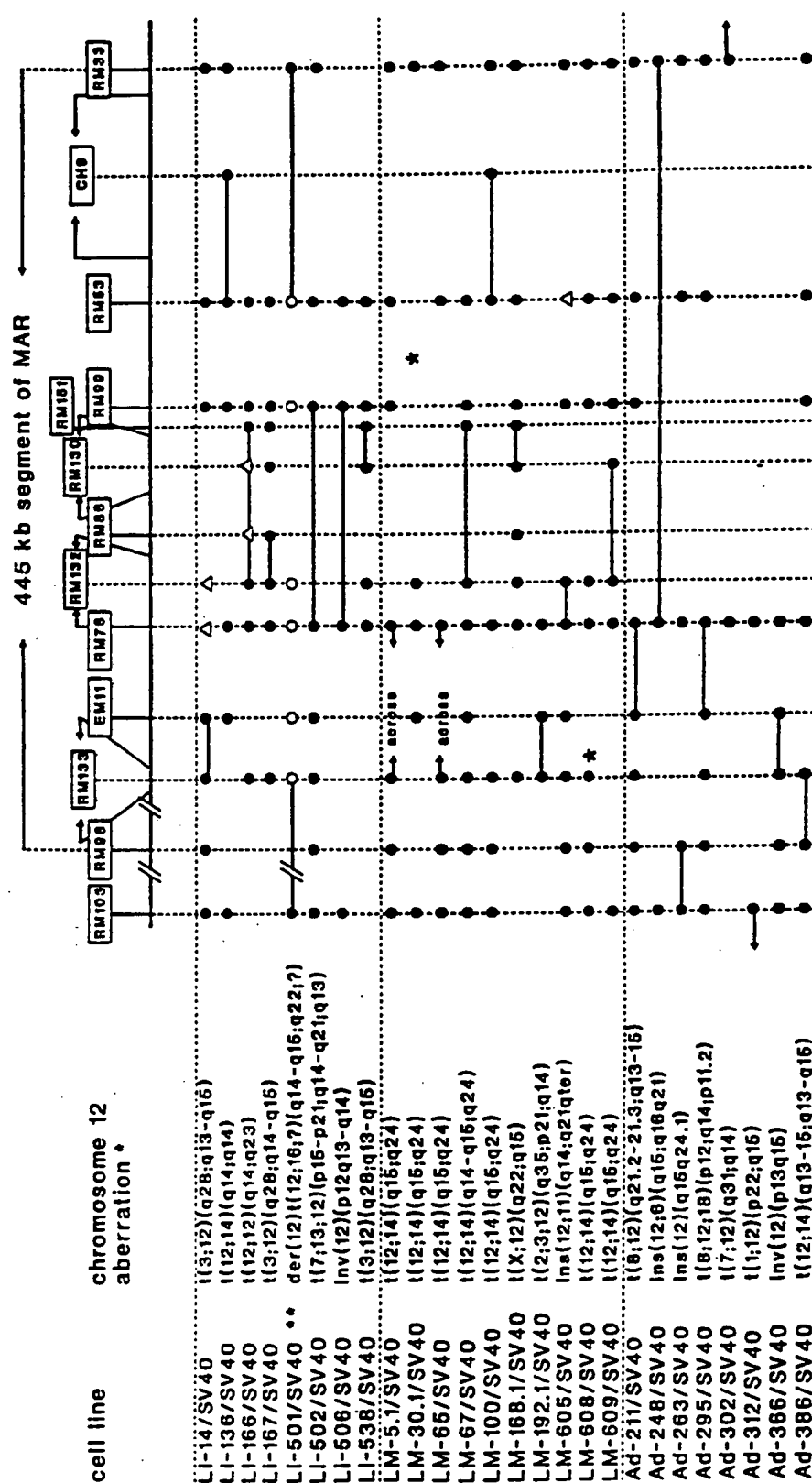# FIG. 4

```
                              primer
CG CTT CAG AAG AGA GGA CGC GGC CGC CCC AGG AAG CAG CAG CAA A        45
   L   Q   K   R   G   R   G   R   P   R   K   Q   Q   Q   K

AA CCA ACC GGT GAG CCC TCT CCT AAG AGA CCC AGG GGA AGA CCC A       90
   P   T   G   E   P   S   P   K   R   P   R   G   R   P   K

AA GGC AGC AAA AAC AAG AGT CCC TCT AAA GCA GCT CAA GAG GAA G      135
   G   S   K   N   K   S   P   S   K   A   A   Q   E   E   A

CA GAA GCC ACT GAA GAA AAA CGG CCA AGG GGC AGA CCT AGG AAA T      180
   E   A   T   E   E   K   R   P   R   G   R   P   R   K   W

HMG           4p
GG GGT GGC CAT TCA GGG CAA CTG GGG CCT TCG TCA GTT GCC CCT T      225
   G   G   H   S   G   Q   L   G   P   S   S   V   A   P   S

CA TTC CGC CCA GAG GAT GAG CTT GAG CAC CTG ACC AAA AAG ATG C      270
   F   R   P   E   D   E   L   E   H   L   T   K   K   M   L

TG TAT GAC ATG GAA AAT CCA CCT GCT GAC GAA TAC TTT GGC CGC T      315
   Y   D   M   E   N   P   P   A   D   E   Y   F   G   R   C

GT GCT CGC TGT GGA GAA AAC GTA GTT GGG GAA GGT ACA GGA TGC A      360
   A   R   C   G   E   N   V   V   G   E   G   T   G   C   T

CT GCC ATG GAT CAG GTC TTC CAC GTG GAT TGT TTT ACC TGC ATC A      405
   A   M   D   Q   V   F   H   V   D   C   F   T   C   I   I

TC TGC AAC AAC AAG CTC CGA GGG CAG CCA TTC TAT GCT GTG GAA A      450
   C   N   N   K   L   R   G   Q   P   F   Y   A   V   E   K

AG AAA GCA TAC TGC GAG CCC TGC TAC ATT AAT ACT CTG GAG CAG T      495
   K   A   Y   C   E   P   C   Y   I   N   T   L   E   Q   C

GC AAT GTG TGT TCC AAG CCC ATC ATG GAG CGG ATT CTC CGA GCC A      540
   N   V   C   S   K   P   I   M   E   R   I   L   R   A   T

CC GGG AAG GCC TAT CAT CCT CAC TGT TTC ACC TGC GTG ATG TGC C      585
   G   K   A   Y   H   P   H   C   F   T   C   V   M   C   H

AC CGC AGC CTG GAT GGG ATC CCA TTC ACT GTG GAT GCT GGC GGG C      630
   R   S   L   D   G   I   P   F   T   V   D   A   G   G   L

TC ATT CAC TGC ATT GAG GAC TTC CAC AAG AAA TTT GCC CCG CGG T      675
   I   H   C   I   E   D   F   H   K   K   F   A   P   R   C

GT TCT GTG TGC AAG GAG CCT ATT ATG CCA GCC CCG GGC CAG GAG G      720
   S   V   C   K   E   P   I   M   P   A   P   G   Q   E   E

AG ACT GTC CGT ATT GTG GCT TTG GAT CGA GAT TTC CAT GTT CAC T      765
   T   V   R   I   V   A   L   D   R   D   F   H   V   H   C

GC TAC CGA TGC GAG GAT TGC GGT GGT CTC CTG TCT GAA GGA GAT A      810
   Y   R   C   E   D   C   G   G   L   L   S   E   G   D   N

AC CAA GGC TGC TAC CCC TTG GAT GGG CAC ATC CTC TGC AAG ACC T      855
   Q   G   C   Y   P   L   D   G   H   I   L   C   K   T   C

GC AAC TCT GCC CGC ATC AGG GTG TTG ACC GCC AAG GCG AGC ACT G      900
   N   S   A   R   I   R   V   L   T   A   K   A   S   T   D

AC CTT TAG ATT CAG TCA CCT GTT CAG CCG GCA CTG AGA AGA ACG A      945
   L   *   I   Q   S   P   V   Q   P   A   L   R   R   T   N

AC ACA AGA AAA AGA TAA GAA ATA CTA GAG TAA AGG CCA TCA AAC T      990
   T   R   K   R   *   E   I   L   E   *   R   P   S   N   Y

                 primer
AC GCG AAA AAA AAA AAA AAA AAA AAA GAT GTC GAC GGA TCC TT        1033
   A   K   K   K   K   K   K   K   D   V   D   G   S   *
```

FIG. 5-1

"GEN3" → partial cDNA sequence

```
GTCACTTTTA TTTGGGGGTG TGGACAGCTG CTTTCCCAGG GGAGTACTTC TTACAGTGGG    60
ATTTCAAGAC AAGATCGGCC TGAAGAAAAA TTATATTTGT ATATTTTTTA AAAAGTGGGA   120
ACTTTGAGGC TCAGAGACAG AGCAGAAGAC AGAACCTGGT CTTCTGATTC CCTGTGTTCT   180
GCTTTTTTCA TTGTTCCACT GGACGCTCAT CAGAGGGAAG ATCTTTTTCC TCAATTGATT   240
CCAACAATGT CTCACCCATC TTGGCTGCCA CCCAAAAGCA CTGGTGAGCC CCTCGGCCAT   300
GTGCCTGCAC GGATGGAGAC CACCCATTCC TTTGGGAACC CCAGCATTTC AGTGTCTACA   360
CAACAGCCAC CCAAAAAGTT TGCCCCGGTA GTTGCTCCAA AACCTAAGTA CAACCCATAC   420
AAACAACCTG GAGGTGAGGG TGATTTTCTT CCACCCCCAC CTCCACCTCT AGATGATTCC   480
AGTGCCCTTC CATCTATCTC TGGAAACTTT CCTCCTCCAC CACCTCTTGA TGAAGAGGCT   540
TTCAAAGTAC AGGGGAATCC CGGAGGCAAG ACACTTGAGG AGAGGCGCTC CAGCCTGGAC   600
GCTGAGATTG ACTCCTTGAC CAGCATCTTG GCTGACCTTG AGTGCAGCTC CCCCTATAAG   660
CCTCGGCCTC CACAGAGCTC CACTGGTTCA ACAGCCTCTC CTCCAGTTTC GACCCCAGTC   720
ACAGGACACA AGAGAATGGT CATCCCGAAC CAACCCCCTC TAACAGCAAC CAAGAAGTCT   780
ACATTGAAAC CACAGCCTGC ACCCCAGGCT GGACCCATCC CTGTGGCTCC AATCGGAACA   840
CTCAAACCCC AGCCTCAGCC AGTCCCAGCC TCCTACACCA CGGCCTCCAC TTCTTCAAGG   900
CCTACCTTTA ATGTGCAGGT GAAGTCAGCC CAGCCCAGCC TCATTATAT GGCTGCCCCT   960
TCATCAGGAC AAATTTATGG CTCAGGGCCC CAGGGCTATA ACACTCAGCC AGTTCCTGTC  1020
TCTGGGCAGT GTCCACCTCC TTCAACACGG GGAGGCATGG ATTATGCCTA CATTCCACCA  1080
CCAGGACTTC AGCCGGAGCC TGGGTATGGG TATGCCCCCA ACCAGGACG CTATTATGAA  1140
GGCTACTATG CAGCAGGGCC AGGCTATGGG GGCAGAAATG ACTCTGACCC TACCTATGGT  1200
CAACAAGGTC ACCCAAATAC CTGGAAACGG GAACCAGGGT ACACTCCTCC TGGAGCAGGG  1260
AACCAGAACC CTCCTGGGAT GTATCCAGTC ACTGGTCCCA AGAAGACCTA TATCACAGAT  1320
CCTGTTTCAG CCCCCTGTGC GCCACCATTG CAGCCAAAGG GTGGCCATTC AGGGCAACTG  1380
GGGCCTTCGT CAGTTGCCCC TTCATTCCGC CCAGAGGATG AGCTTGAGCA CCTGACCAAA  1440
AAGATGCTGT ATGACATGGA AAATCCACCT GCTGACGAAT ACTTTGGCCG CTGTGCTCGC  1500
TCTCGAGAAA ACGTAGTTGG GGAAGGTACA GGATGCACTG CCATGGATCA GGTCTTCCAC  1560
GTGGATTGTT TTACCTGCAT CATCTGCAAC AACAAGCTCC GAGGGCAGCC ATTCTATGCT  1620
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FIG. 5-2

```
GTGGAAAAGA AAGCATACTG CGAGCCCTGC TACATTAATA CTCTGGAGCA GTGCAATGTG   1680

TGTTCCAAGC CCATCATGGA GCGGATTCTC CGAGCCACCG GGAAGGCCTA TCATCCTCAC   1740

TGTTTCACCT GCGTGATGTG CCACCGCAGC CTGGATGGGA TCCCATTCAC TGTGGATGCT   1800

GGCGGGCTCA TTCACTGCAT TGAGGACTTC CACAAGAAAT TTGCCCCGCG GTGTTCTGTG   1860

TGCAAGGAGC CTATTATGCC AGCCCCGGGC CAGGAGGAGA CTGTCCGTAT TGTGGCTTTG   1920

GATCGAGATT TCCATGTTCA CTGCTACCGA TGCGAGGATT GCGGTGGTCT CCTCTCTGAA   1980

GGAGATAACC AAGGCTGCTA CCCCTTGGAT GGGCACATCC TCTGCAAGAC CTGCAACTCT   2040

GCCCGCATCA GGGTGTTGAC CGCCAAGGCG AGCACTGACC TTTAGATTCA GTCACCTGTT   2100

CAGCCGGCAC TGAGAAGAAC GAACACAAGA AAAAGATAAG AAATACTAGA GTAAAGGCCA   2160

TCAAACTACG CGATAGTCTC TGTTCTTCAT CTGCTATTAA CCTTGCCTTA GAAACACATA   2220

AATTATGAGA TTTTTTTTTA AAAGTTGTTA CCAAATACAC ATTTCACATT GAATCATGTA   2280

GGATCTTGAT GGGCCTTTGT TCCCAAGGAC TTCCACATTT TTGCACAGAT TATGCTCCAT   2340

CCCTTCACTT CTGCATTCCT GTAACTTTTA ATCCCTATGT TTGTCTCACT TTTCATCTGG   2400

TTGAATGGCT TTTCTTAGTG TGGTATTTGC TGTCACATAG TTTTTTCCTG GGTGAGTCTG   2460

CCAACTCACA GGTGCTTTTA GGCTTGAAAT CTCCATCCTA TCATTTCCGT TTTGCCTGTG   2520

ACTGTAAAGA GTAGCCATTC TTTTCCCATG TATTGAAGAG GATATTCTTC TCTTGCTTTA   2580

TACTACTCAC GTCCTTGGGG AGGGAAATGC ACAATTTTTT TTTGTTAGGC TGTAAAGAAT   2640

TTAAGCTGTA AATTACATAA GTTAGAACAA GCCCAAATTT AATTTGCAAC CATCAGAATT   2700

CAGAATCTAT AGTGACCAGT GATCAAGGCT AATTGGAAAA GAGTTATCGG CCCATAGCTA   2760

ATAAGTAGTG ACAGACAACC AAGCTTCAAT ATTTTTCTAA AGAAATTACA GGTGGGATAT   2820

GCTAGAAAAG GCATTTTGGG GTTATGTTTA AAAAAACATT ATTGTCCCAC AATATTACCT   2880

TAAGATTTTT CTTTTCCGCA CTACCTGAAC ATTGTAATAC AGACAAACTT GATTCTTCT   2940

AGAAGATAAC ATTTTCAATA CTGTCCCACT TCTCATCTTA AAAATATTGT CATGTTTATT   3000

CTAATATCCA ACGCAACTAT CAAAATTGCC TTTTTCTCTA GAGGATGAAG GCTGTGAAAA   3060

AACCGTTCAA ATTCTCTTCT TTTTCTTTTT TATTACCAGG TCCATTTTGC CTGACAATTG   3120

CAAATCAGAG CATACAAAAT AAAACTGTGC AGTTTTGTTT GGTTTACTTT CAAAAGAGTA   3180

GAAAGCTTGA AAAGATTCTG AAACCACAGT TTCATTATTC TCATAATCCT TCTGCAACTG   3240

AAATTACATA TTGCAGGAGA CATTTTCATA TCATCAATGT GACATTTACA CCACACTTTC   3300

AAAGACAATC ACTGAAACAA AAATTGTCTT TATCAGCTAA AAATATGCAG AATCTCTGCC   3360
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FIG. 5-3

```
TAGAATCTTT ATTCAAACTT TTATTAGCCA GTGAAACACT TGCTTGCCAA CTGCCAAGCC    3420
ATACTTATTA AGTTCGAACA TGTTTCACTT AAGGAGAGAC ACCTAGCTTA GTCATGGCAA    3480
GTTGCCATTT TGTAAACTAA GGATTTTGGA CTGAGATTTC TTAAATCTTT CTTCAAATCT    3540
CCCACAAGTA TATACTTTTA AATTATGGAG TATTTTAAGT CTACAAAAAG GTATAAATAA    3600
TAATATAATG AATTCCTATA TACCTAATAC CCAGTTTAAG ACACCAAATA TAACAAGTAT    3660
AATTACATCC TCCAATGTAC CGTTTCCTTA TTCCACAGAT ATCTTTTTCA TTATTGTGAA    3720
GTGATGTTCA GATTTCTAGT TTTTTTTTCT AGTTTTTAAT TTTAACATCA GAACTGAAAT    3780
AAAAAATTAT GGATACGTGT TTTGAATTGC AAACTATTCC TCAGGAATTC CAATTAAATT    3840
TATTTTACTT GAATAGGAAT GATCATAAAA GTGATTCTTT TTTTGTGACT AGAAATTCTT    3900
AAGCCGATGG TCACTATAGC TCATCCTTAA TGTATGGCTC ATTTGCTTTT GTCACTAAAC    3960
GGTTTTGTGT TAGAACCACC AAAATTATAG CTTTTAAGAG CTTCCTTTGA CCACTGTCTT    4020
TTTCTTACCC TACTTCTCTT ATCTTTGATC GTATATTTCT CATAATGTGA AATATGATGA    4080
GATTCACTTA GGGGCAGCAT GTTAGTTTTG GGAGGCAATG TCAACTGTGT CTCTGAATTC    4140
CTGTCTTCCA AATTGAAGCC AGACCATGCT GATGACCTCA AGTAGCACTG ACTATTTGAC    4200
AATAGGGCTG ATAATGTAAT CGGCTTGAAT TTTGACTTAG TAACTTTTTA TGTAATACTT    4260
TCGGAGAAAT TCTCTTTAGG ACAAAGCAGA GAGTCCAATT TATTGAGGGA TAGATTGTAT    4320
CTC                                                                  4323
```

FIG. 6

MSHPSWLPPKSTGEPLGHVPARMETTHSFGNPSISVSTQQPPKKFAPVVAPKPKY

NPYKQPGGEGDFLPPPPPPLDDSSALPSISGNFPPPPPLDEEAFKVQGNPGGKTL

KERRSSLDAEIDSLTSILADLECSSPYKPRPPQSSTGSTASPPVSTPVTGHKEMV

IPNQPPLTATKKSTLKPQPAPQAGPIPVAPIGTLKPQPQPVPASYTTASTSSRPT

FNVQVKSAQPSPHYMAAPSSGQIYGSGPQGYNTQPVPVSGQCPPPSTRGGMDYAY

IPPPGLQPEPGYGYAPNQGRYYEGYYAAGPGYGGRNDSDPTYGQQGHPNTWKREP

GYTPPGAGNQNPPGMYPVTGPKRTYITDPVSAPCAPPLQPKGGHSGQLGPSSVAP
                                                LIM 1
SFRPEDELEHLTKKMLYDMENPPADEYF GRCARCGENVVGEGTGCTAMDQVFHVD
                                                LIM 2
CFTCIICNNKLRGQPFYAVEKKAYCEPCYINTLEQCNVCSKPIMERIILRATGKAY
                                                LIM 3
HPHCFTCVMCHRSLDGIPFTVDAGGLIHCIEDFHKFAPRCSVCKEPIMPAPGQE

ETVRIVALDRDFHVHCYRCEDCGGLLSEGDNQGCYPLDGHILGKTCNSARIRVLT

AKASTDL*

FIG. 7-1

BASE COUNT   1119 a   988 c   791 g   1169 t
ORIGIN

```
   1 cttgaatctt ggggcaggaa ctcagaaaac ttccagcccg ggcagcgcgc gcttggtgca
  61 agactcaggaa gctagcagcc cgtccccctc cgactctccg gtgccgccgc tgcctgctcc
 121 cgccaccctа ggaggcgcgg tgccacccac tactctgtcc tctgcctgtg ctccgtgccc
 181 gaccctatcc cggcggagtc tcaccatcct cctttgcttt ccgactgccc aaggcacttt
 241 caatctcaat ctcttctctc tctctctctc tctctctgtc tctctctctc tctctctctc
 301 tctctctctc gcagggtggg gggaagaggа ggaggaattc tttccccgcc taacatttca
 361 agggacauaa ttcactccaa gtctcttccc tttccaagcc gcttccgaag tgctcccggt
 421 gccccgcaacl cctgatccca accgcgcgaga ggagcctctg cgacctcaaa gcctctcttc
 481 cttctccctc gcttcectcc tcctcttgct acctcaacct ccaccgccac ctccacctcc
 541 ggcacccacc cacccggcggc gccgcuaccg gcagcgcctc ctcctctcct cctcctcctc
 601 ccctcttctc ttttggcag ccgctggacg tccggtgttg atggtggcag cggcggcagg
 661 ctaagcaaca gcagccctcg cagcccgcca gctcgcgctc gccccgccgg cgtcccgagc
 721 cctatcaact catctcccga aaggtgctgg gcagctccgg ggcggtcgag gcgaagcggc
 781 tgcagcggcg gtagcggcgg cgggaggcag gatgagcgca cgcggtgagg gcgcgggcca   START
 841 gccgtccact tcagcccagg gacaaactgc cgcccaagcg cctcagaaga gaggacgcgg
 901 ccgcccaagg aagcagcagc aagaaccaac cggtgagccc tctcctaaga gacccagggg
 961 aagacccaaa ggcagcaaaa acaagagtcc ctctaaagca ggtcaaaaga aagcagaagc
1021 caactggagaa aaacggccaa gaggcagacc taggaaatgg acacaacaag ttgttcagaa
1081 gaagcctgct caggaggaaa ctgaagagac atcctcacaa gagtctgcaa aagaggacta   STOP
1141 ggggcggcaa cgttcgcttt ctacctcagc agcagttgga tatttgaag ggagcagaca
1201 ctgcagtgac cacttattct gtattgccat ggtctttcca cttttcatctg ggtgggtg
1261 ggctggggtg gggagcgggg gggtcgggtc gggagaaatc acataacctt aaaaaggact
1321 atattaatca ccttccttgt aatccccttca cagtccaagg tttagtgaaa aactgctgta
1381 aacacagggg acacagctta acaatgcaaac ttttaattac tgttttcttt tttcttaacc
1441 tactaatagt ttgttgatct gataagcaag agtgggcggg tgagaaaaac cgaattgggt
1501 ttagtcaatc actgcactgc atgcaaacaa gaaacgtgtc acacttgtga cgtcgggcat
1561 tcatatagga agaacgcggt gtgtaacact gtgtaacact caaatacccac cccaaccccac
1621 tccctgtagt gaatccctctg tttagaacac caaagataag gactagatac tactttctct
1681 tttttgtata atcttgtaga cacttccttg atgattttta acttttttatt tctaaatgag
1741 acgaaatgct gatgtatcct ttcattcago taacaaacta gaaaaggtta tgttcatttt
1801 tcaaaaaggg aagtaagcaa acaaatattc ccaactcttc tatttatgga tatcacacat
1861 atcaggcagga gtaataaatt tactcacagc acttgttttc aggacaacac ttcattttca
1921 gguaalctac ttcctacaga gccaaaatgc catttagcaa taaataacac ttgtcagcct
1981 cagagcattt aaggaaacta gacaagtaaa attatccttct ttgtaattta atgaaaggt
2041 acaacagaat aatgcatgat gaacctcaacl aattatgagg tggggaggago gaaatctaaa
2101 tttctttttgc tatagttata catcaatttа aaaagcaaaa aaaaaaaggg gtgggcaatc
2161 trtctctgtg tgtttctctc tctctctccc tctccctctc tcttttcatg tgtatcagtt
2221 tacataaag acctgaatac cacttacctc aaattaagca tatgtgttac ttcaagtaat
2281 acgtttttgac ataagatggt tgacaaaggt gctttctcttc gycttgagtt caccatctct
2341 tcattcaaac tgcactttta gccagagatg caatatatcc ccactactca atactacctc
2401 tgaatgttac aacgaattta cagtctagta cttattacal gctgctatac acaagcaatg
2461 caagaaaaaa acttactggg taggtgatto taatcatctg cagttctttt tgtacactta
2521 attacagtta aagaagcaat ctccttactg tgtttcagca tgactatgta tttttctatg
2581 lttttttaat taaaaatttt taaaatactt gtttcagctt ctctgctaga tttctacatt
2641 аacttgaaa ttttttaacc aagtcgctcc taggttctta aggataatttt tcctcaatca
2701 cactacacat cacacaagat ttgactgtaa tatttaaata ttaccctcca agtctgtacc
2761 tcaaatgaat tctttaagga yatggactaa ttgacttgca aagacctacc tccagactto
2821 aaaaggaatg aacttgttac ttgcagcatt catttgtttt ttcaattgttt gaaatagtto
2881 aaactgcagc taaocctagt caaaactatt tttgtaaaag acatttgata gaaaggaaca
2941 cgtttttaca tacttttgca aaataagtaa ataataaata aaataaagcc aacttcaaa
3001 gaacttgaag ctttgtaggt gagatgcaar aagecctgct tttgcataat guaatcaaaa
3061 atatgtgttt ttaagattag ttgaatataa gaaaatgctt gacaaatatt ttcatgtatt
3121 ttacacaaat gtgatttttg taalatgtct caaccagatt tattttaaac gcttcttatg
3181 tagagttttt atgcctttct ctcctagtgа gtgtgctgac tttttaacat ggtattatca
3241 actgggccag gaggtagttt gtcatgacgg cttttgtcag tatggctttt agtactgaag
3301 ccaaatgaaa ctcaaaacca tctctcttcc agctgcttca gggaggtagt ttcaaaaggcc
3361 acatacctct ctgagactgg cagatogctc actgttgtga atcaccaaag gagctatgga
3421 gagaattaaa actcaacatt actgttaaat gtggcgttaaa taagcaaata aacagtggct
3481 cataaaaata aaagtcggat trcatatctt tggatgggcc ttttagaaac ctcattggcc
3541 agctcaataaa atgcaagcaa ttgatcatgt tggccaaaca tggtgcaccg agtgatttcc
3601 atctctggta aagttacact tttatttcct gtatgttgta caatcaaaac acactactac
3661 ctcttaagtc ccagtataac tcatttttoa tactgaaaaa aacagcttgt cgccaatgga
```

FIG. 7-2

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3721 acagtaagaa aataataaaa tttttatata tatagtttat ttttgtggga gataaatttt
3781 ataggactgt tctttgctgt tgttggtcgc agctacataa gactggacat ttaactttto
3841 tacaatttct gcaagttagg tatgtttgca ggagaaaagt atcaagacgt ttaactgcag
3901 ttgactttct caetgttcct ttgagtgtct tctaactttaa ttcttcgttc tttatgtaga
3961 attgctgtct atgattgtac tttgaatcgc ttgcttgttg aaaatattto tctagtgtat
4021 tatcactgtc tgttctgcac aataaacata acagcctctg tgatcce
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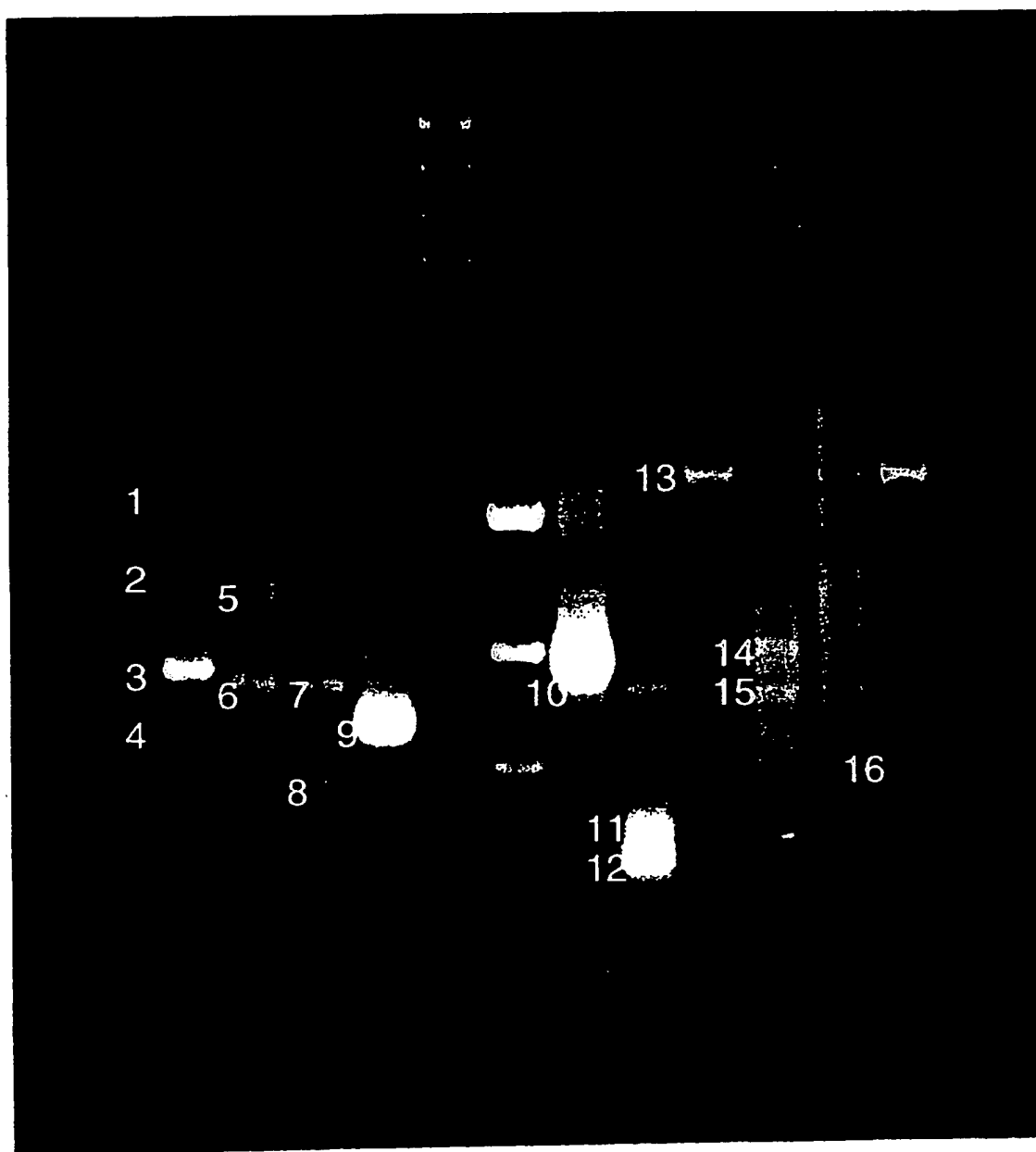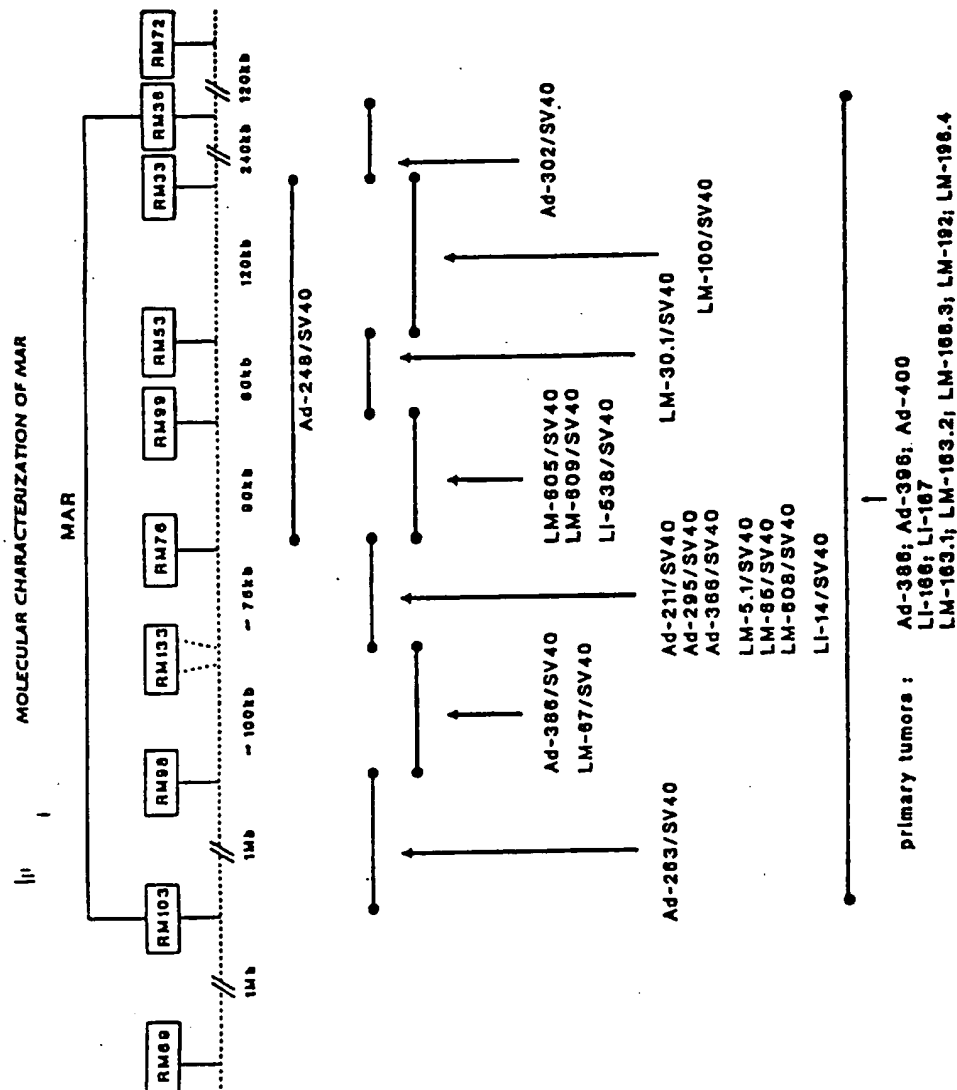
FIG. 8
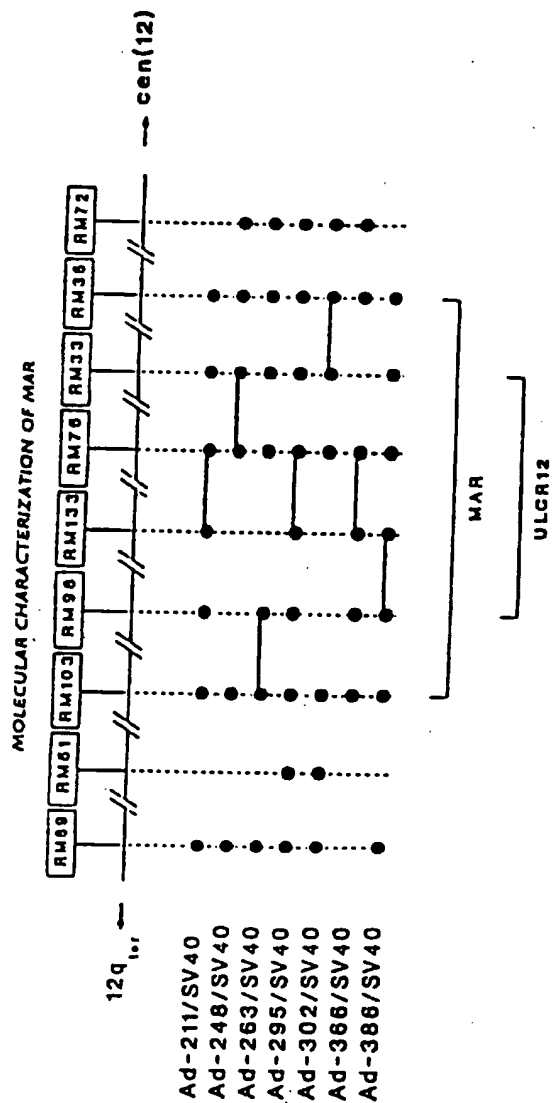
Figure 1 to Annex 1
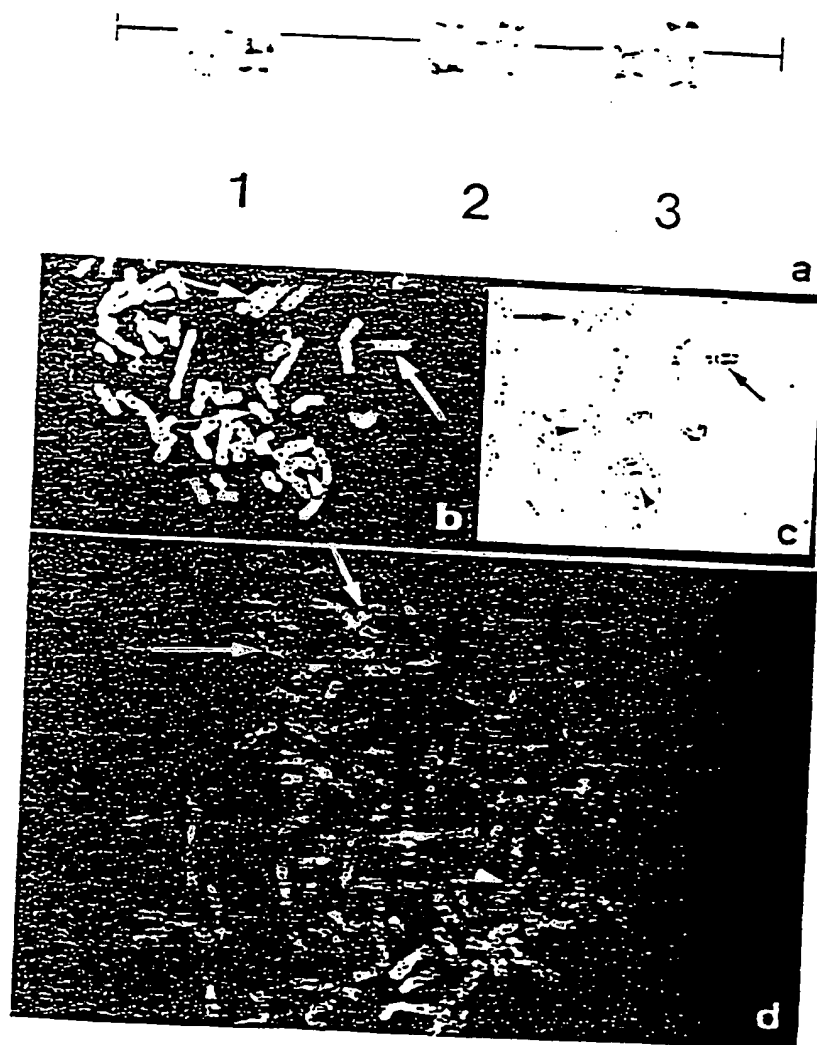
Figure 2 to Annex 1

Figure 3 to Annex 1

Figure 1 of Annex 2
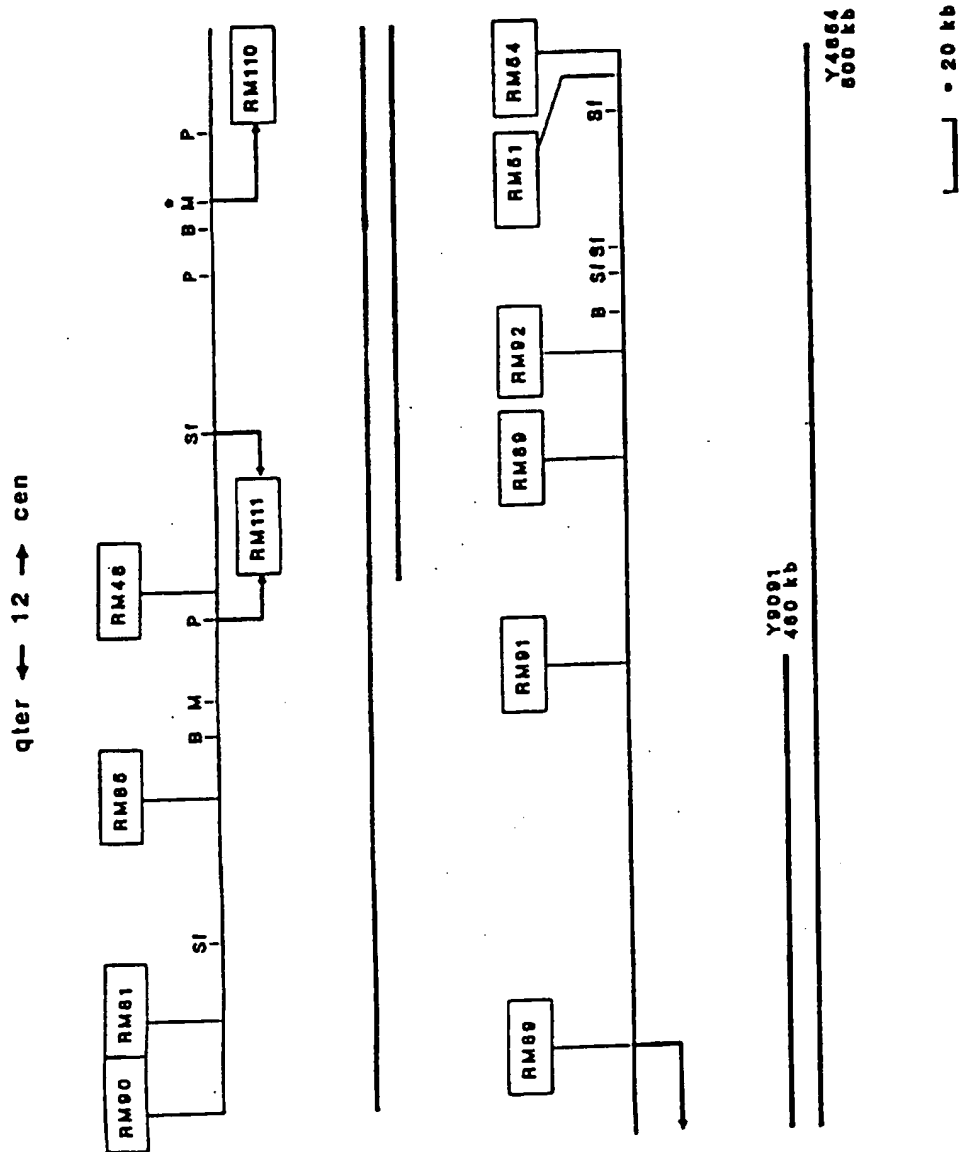
Figure 2 of Annex 2
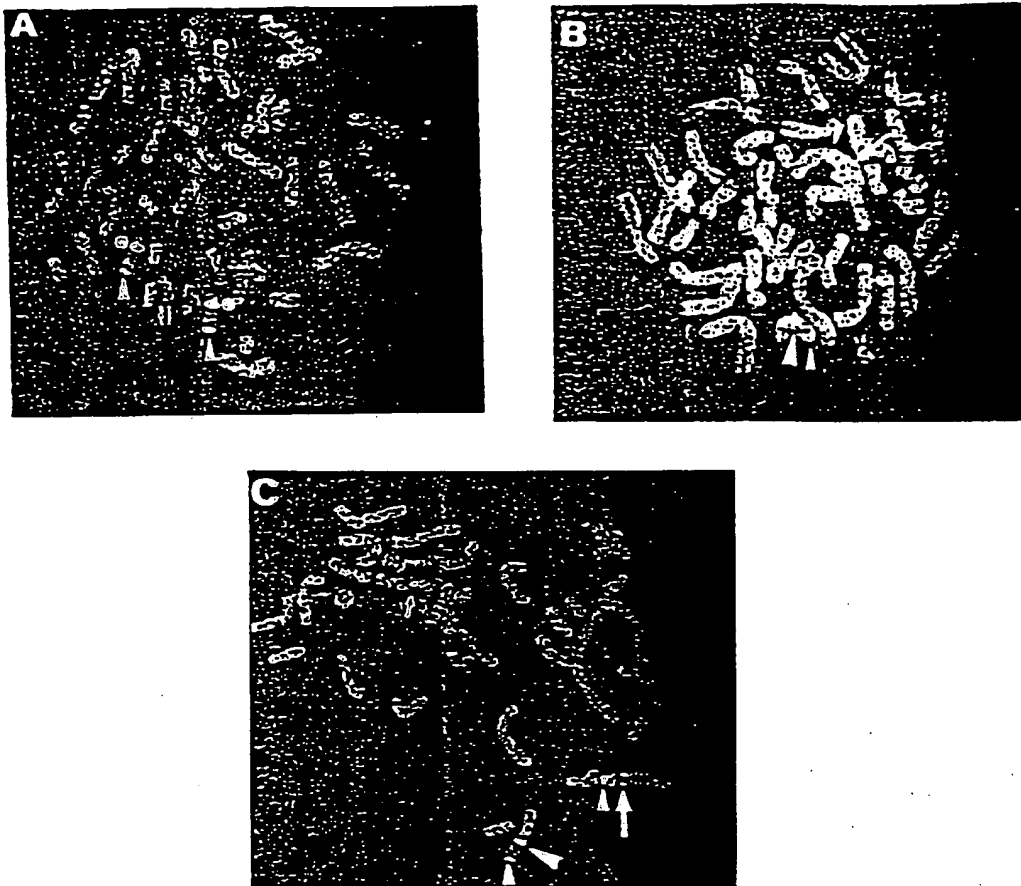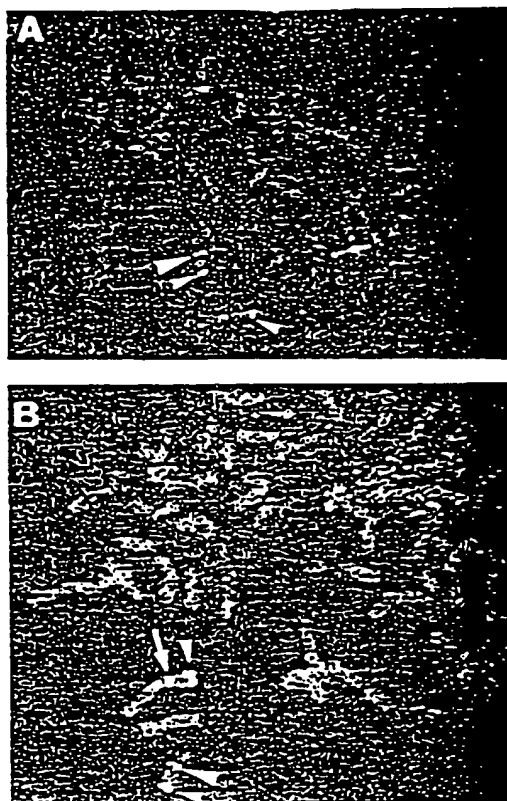
Figure 3 of Annex 2

Figure 4 of Annex 2
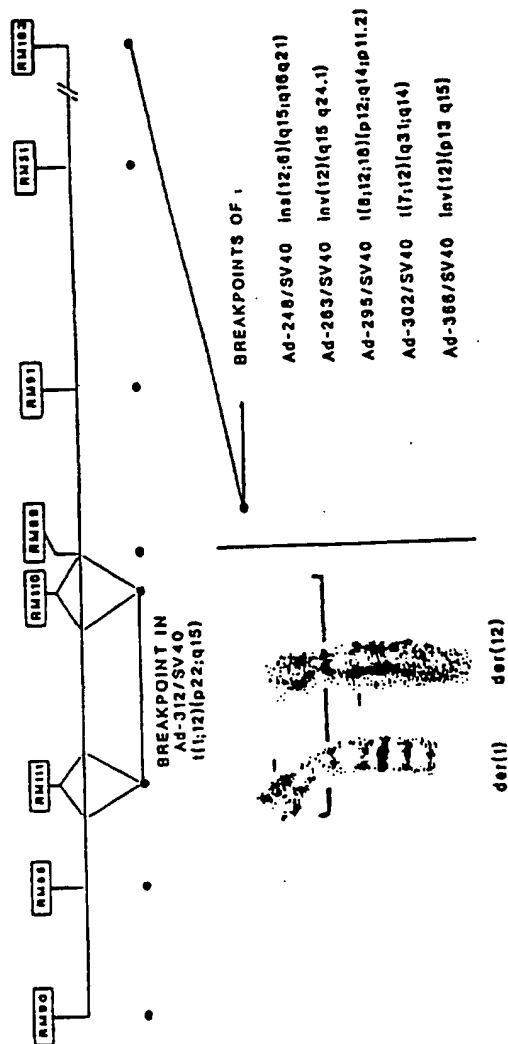
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/12   C12N15/11   C07K14/47   C12Q1/68   C07K19/00
C07K16/18   A61K38/17   A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C07K   C12Q   A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 122, no. 7, 13 February 1995 Columbus, Ohio, US; abstract no. 73586t, SCHOENMAKERS,ERIC ET AL.: "Identification,molecular cloning and characterization of the chromosome 12 breakpoint cluster region of uterine leiomyomas." page 267; XP002005567 see abstract & GENES,CHROMOSOMES CANCER , vol. 11, no. 2, 1994, pages 106-118,	1,2,4-7

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[X] Further documents are listed in the continuation of box C.   [ ] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 June 1996	12.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Delanghe, L

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH, vol. 19, no. 24, 1991, OXFORD GB, pages 6793-6797, XP002005564 G.MANFIOLETTI ET AL.: "cDNA cloning of the HMGI-C phosphoprotein ,a nuclear protein associated with neoplastic and undifferentiated phenotypes." see the whole document ---	1,2,4-7
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 201, no. 1, 30 May 1994, ORLANDO, FL US, pages 63-70, XP002005565 UMESH A. PATEL ET AL.: "Expression and cDNA cloning of human HMGI-C phosphoprotein." see the whole document ---	1,2,4-7
X	DATABASE WPI Section Ch, Week 8735 Derwent Publications Ltd., London, GB; Class B04, AN 87-245773 XP002005570 & JP,A,62 166 897 (TOYO SODA MFG KK) , 23 July 1987 see abstract ---	6
P,X	NUCLEIC ACIDS RESEARCH, vol. 23, no. 21, 1995, OXFORD GB, pages 4262-4266, XP002005566 KAI-YIN CHAU ET AL.: "The gene for the human architectural transcription factor HMGI-C consists of five exons each coding for a distinct functional element." see the whole document ---	1,2,4-7
P,X	CHEMICAL ABSTRACTS, vol. 123, no. 1, 3 July 1995 Columbus, Ohio, US; abstract no. 6842f, VAN DE VEN,WIM J.M. ET AL.: "Molecular characterization of MAR,a multiple aberration region on human chromosome segment 12q13-q15 implicated in various solid tumors." page 721; XP002005568 see abstract & GENES,CHROMOSOMES CANCER, vol. 12, no. 4, 1995, pages 296-303, ---	1,2,4-7

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	Inter⁻ ·l Application No
	PCT/LP 96/00716

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	CHEMICAL ABSTRACTS, vol. 123, no. 15, 9 October 1995 Columbus, Ohio, US; abstract no. 195315v, SCHOENMAKERS,ERIC ET AL.: "Recurrent rearrangements in the high mobility group protein gene,HMGI-C,in benign mesenchymal tumors." page 885; XP002005569 see abstract & NAT.GENET. , vol. 10, no. 4, 1995, pages 436-444, -----	1

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